UNIVERSITE DE GENEVE
Département de Biochimie

FACULTE DES SCIENCES
Professeur B.W. FULPIUS

EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS: POSSIBLE ROLE OF ANTIACETYLCHOLINE RECEPTOR ANTIBODIES IN THE APPEARANCE OF THE NEUROMUSCULAR BLOCKADE

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de l'Université de Genève
pour obtenir le grade de docteur en biologie médicale

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ABBREVIATIONS

MG	Myasthenia gravis
EAMG	experimental autoimmune myasthenia gravis
AcCh	acetylcholine
AcChR	acetylcholine receptor
AcChE	acetylcholine esterase
α-Bgt	alpha-Bungarotoxin
α-T	cobra alpha-neurotoxin
HTX	histrionicotoxin
d-TC	d-tubocurarine
carbachol	carbamylcholine
C_6	hexamethonium
MBTA	4-(N-maleimido)-2-benzyltrimethylammonium
ICM	ion conductance modulator
AP	action potential
EPP	endplate potential
MEPP	miniature endplate potential
IgG	gamma-immunoglobulin
SARIG	sheep anti-rabbit IgG
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
nmole	nanomole = 10^{-9} mole
pmole	picomole = 10^{-12} mole

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FOREWORD

Myasthenia gravis (MG) is a rare neuromuscular disease of man (2-4 cases per 100.000 persons {Havard, 1977}) characterized clinically by muscle fatigability which increases with exertion and improves with rest. Electrophysiological studies have shown that the clinical symptoms are due to a postsynaptic neuromuscular blockade resembling that produced by curare (Grob et al. 1976). Light and electron micrographs of myasthenic neuromuscular junctions show enlarged synaptic clefts, a decreased number of postsynaptic folds (Engel et al. 1976) and a 80% decrease in the number of acetylcholine receptors (AcChR) as visualized by labeling with ^{125}I-α-bungarotoxin (α-Bgt) (Fambrough et al. 1973).

In recent years, auto-antibodies against AcChR have been found in the sera of 87% of myasthenic patients (Lindstrom et al. 1976e) and in the blood of babies with neonatal myasthenia gravis (Keesey et al. 1977). The presence of these antibodies in addition to antibodies directed against skeletal muscle striations, cell nuclei or thyroid gland, which are present in a certain percentage of myasthenic sera (Meyer 1976); the high frequency of thymic abnormalities observed (5-15% of the patients have a thymoma, 70-80% a thymic hyperplasia {Elias and Appel,1976}) and the frequent association of myasthenia gravis with other autoimmune disorders, favor an autoimmune etiology of MG (Simpson 1960, Havard 1977).

In addition, some genetical predisposition exists: (i) HLA typing has revealed a prevalence of the HLA-B8 antigen marker among young patients who have a thymic hyperplasia (Pirskanen 1977), (ii) HLA-B8 has been reported to be increased in several autoimmune diseases (Pirskanen 1977) and (iii) cases of familial occurrence among myasthenic patients have been observed (Herrmann 1971, Pirskanen 1977).

We have chosen to analyse the possibility that anti-AcChR antibodies act as curare-like agents in the genesis of the neuromuscular blockade observed in MG. To do so, we have used an experimental model of MG: experimental autoimmune myasthenia gravis (EAMG) (Patrick and Lindstrom, 1973a, Sugiyama et al. 1973). This experimental disease can be induced in different animals (rabbits, rats, guinea pigs, etc) (Heilbronn, 1976) by immunization with AcChR isolated from electric organs of fish (Patrick and Lindstrom, 1973a, Sugiyama et al. 1973) or denervated skeletal muscle (Granato et al. 1976). The animals produce high titers of antibodies against the immunizing antigen and develop a flaccid paralysis similar in many respects to that observed in humans (Lennon, 1976).

In the present work I am giving first an introduction on the neuromuscular junction, AcChRs and anti-AcChR antibodies by means of data from the literature. Then I am giving a list of the methods available in order to analyse the effect of anti-AcChR antibodies on AcChR. The methods used and the results are either described in the text or in the publications included after the unpublished results.

INTRODUCTION

The primary function of nerve cells is that of receiving, integrating, carrying and transmitting messages. They form the communication system between the external environment and the organism and between distant points of the organism (Nachmansohn 1975). The structure responsible for the transmission of messages between excitable cells is called a synapse (Katz 1966). In the case of the contact between nerve and muscle cells, the synapse is called neuromuscular junction.

Neuromuscular junction

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Figure 18.2 Diagram showing detailed view of the neuromuscular junction. SC: terminal Schwann cells, GU: synaptic gutters, JF: subneural folds of the postsynaptic membrane, NE: cross section of the terminal nerve branches at the motor end plate. (Photograph supplied by Dr. Keith R. Porter.)

(taken from Tu, 1977)

When a nerve cell is stimulated, the impulses are propagated electrically to the nerve terminals as action potentials (AP). The arrival of these impulses causes the release of a chemical transmitter from the nerve terminal into the synaptic cleft

(the synaptic cleft of rat diaphragm has a width of about 600 Å, Fertuck and Salpeter, 1974). In 1936, Dale et al. showed that the chemical transmitter was acetylcholine (AcCh). On the postsynaptic muscle membrane AcCh produces a transient conductance change by opening ionic channels to small ions like sodium, potassium and calcium. A localized depolarization develops, the endplate potential (EPP). When the EPP reaches a certain threshold, it induces an action potential which propagates along the muscle fiber. Consequently, calcium ions will be released into the sarcoplasmic reticulum and cause actin and myosin to interact. This will finally lead to muscle contraction. In the synaptic cleft, AcCh is hydrolyzed by acetylcholine esterase (AcChE). This limits the duration of membrane depolarization (Barnard, 1974). AcChE is highly concentrated at the postsynaptic membrane (Koelle et al., 1949). There are 0.3 to 4 x 10^7 AcChE molecules per endplate in different adult muscles (Barnard et al., 1975). Recently, McMahan et al. (1978) demonstrated in frog cutaneous pectoris muscle that AcChE is associated with the basal lamina of the neuromuscular junction.

In 1950, Fatt and Katz showed that, in the absence of any stimulation of the nerve, the junctional region of the muscle was the site of spontaneous discharges of minute endplate potentials (later called miniature endplate potentials {MEPP}). These small depolarizations of about 0.5 mV occur randomly. This led to the idea that AcCh was secreted from the nerve terminals in discrete multimolecular packets. When a few years later presynaptic vesicles were discovered in nerve terminals, del Castillo and Katz (1954) proposed that these vesicles were the structural units responsible for the "quantal release" described above. In 1964, Whittaker and De Robertis showed by electron microscopy and chemical studies that AcCh was stored in the vesicular compartments of presynaptic endings. According to recent studies (Hartzell et al., 1976), every nerve ending of rat diaphragm contains 500-1000 vesicles, each containing about 10.000 AcCh molecules (= one quantum). The impulse at the nerve terminal causes, through Ca^{++} influx

(Rahamimoff, 1976), 300-500 vesicles to fuse with the presynaptic membrane at specialized release sites (Barnard et al.,1975). The AcCh content is liberated into the synaptic cleft by a process of exocytosis (Heuser and Reese, 1973)*.

Localization and function of the AcChR

The concept of receptors on cell surfaces responsible for the recognition and binding of chemical compounds was postulated in 1907 by Langley. In 1955, Altamirano et al., showed that the depolarization of the postsynaptic membrane induced by AcCh was due to the binding of AcCh to a site distinct from the active site of AcChE. On the electric organ of the marine ray *Torpedo*** they showed that some compounds may block synaptic transmission while having almost no effect on the AcChE. This was, however, only evidence for two distinct binding sites for AcCh and not for the presence of a receptor molecule distinct from the AcChE molecule.

In 1971, Changeux et al. were able to show that the receptor for AcCh (AcChR) was a molecule distinct from the AcChE. This was made possible by the discovery of specific neurotoxins in the venom of Elapid snakes (Chang and Lee, 1963). These neurotoxins, like curare, block the depolarizing effect of AcCh on neuromuscular junctions and electric organ cells without affecting AcChE activity. The neurotoxins are small proteins (Lee 1972,

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* According to Nachmansohn (1975) there is still no experimental evidence for the vesicle hypothesis of synaptic transmission proposed by del Castillo and Katz (1954). It is not clear how AcCh can get into the vesicles because there is no acetyl-transferase within the vesicles. Furthermore, the mechanism of release of AcCh from the vesicles is still undecided. Nachmansohn believes that the AcCh is associated with a storage protein within the cytoplasm. According to Kaufmann (1977) the cytoplasmic AcCh is liberated upon nerve stimulation and not the AcCh present in the vesicles.

** Electric organs of fish like the marine ray *Torpedo* and the electric eel *Electrophorus electricus* are cholinergic systems with an embryologic origin similar to that of skeletal muscle (Feldberg and Fessard, 1942, Bennett 1970).

Tu 1977) consisting of a single polypeptide chain cross-linked with 4 or 5 disulfide bonds.

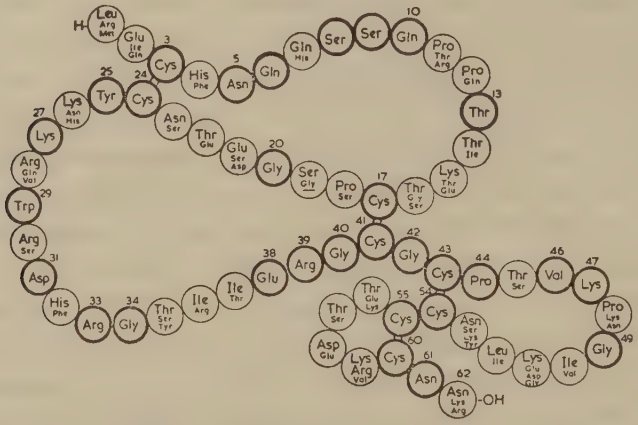

FIG. 1. Common Structure of Sea Snake and Cobra Neurotoxins (Type I). Sea snake NTs: Erabutoxins a & b from *Laticauda semifasciata* (50). Type 1 cobra NTs: Cobrotoxin from *Naja naja atra* (49), Toxin α from *N. nigricollis* (48), Toxin α from *N. haje haje* (36), Toxin δ from *N. nivea* (33), Toxin β from *N. nivea* (33), and Toxins II & IV from *Hemachatus haemachatus* (46). Heavy rings denote amino acid residues common to all of these NTs.

(taken from Lee, 1972)

Their isoelectric point is above 9. They are divided into two groups differing in their amount of amino acid residues and number of disulfide bonds: type I neurotoxins consist of a polypeptide chain made of 61 or 62 amino acid residues cross-linked with 4 disulfide bonds (e.g. cobrotoxin from *Naja naja atra* and erabutoxin from *Laticauda semifasciata*). Type II neurotoxins consist of 71 or 72 amino acid residues with 5 disulfide bonds (e.g. α-toxin from *Naja naja siamensis*). α-Bungarotoxin (α-Bgt) from the Krait venom *Bungarus multicinctus* consists of 74 amino acid residues and 5 disulfide bridges. Its amino acid composition, primary structure and mode of neuromuscular blocking action is very similar to that of type II neurotoxins.

The kinetic properties of these neurotoxins are of interest: their dissociation half-time is in the range of hours. For ^{3}H-α-neurotoxin from *Naja naja siamensis* this half-time is about 83 hours for a high-affinity complex (RT') and 3 hours for a more rapidly dissociating species (RT) (Maelicke et al., 1977). This slow dissociation rate has allowed the successful use of affinity chromatography to purify AcChR from *Electrophorus electricus* electric organ (Changeux et al., 1971). The dissociation constant K_D at equilibrium is of 3.3×10^{-11}M for the high affinity complex and of 6×10^{-10}M for the low affinity complex. Thus, neurotoxins are extremely potent agents: 2-3 µg of purified toxin are sufficient to kill a mouse.

α-Bgt labeled with ^{125}I was used to measure the amount of AcChR present in electric organs of fish and in skeletal muscle. It is expressed as the number of moles of labeled neurotoxin bound, assuming that one AcChR molecule binds one toxin molecule. *Torpedo marmorata* or *californica* electric organ contains 1 to 2 nmoles of AcChR per gram wet tissue. The AcChR represents about 20% of the total electric organ protein (Cohen and Changeux, 1975). By comparison, *Electrophorus electricus* electric organ contains 50 to 100 pmoles AcChR per gram tissue, innervated mammalian skeletal muscle 1 to 8 pmoles and denervated skeletal muscle* 50 to 100 pmoles (for a review, see Fulpius, 1976).

125I-α-Bgt was also used to localize and quantitate AcChRs in the postsynaptic membrane of skeletal muscle by electron microscopy and autoradiography: endplates of various muscles were shown to contain 2 to 8×10^{7} AcChR molecules (Barnard et al., 1975). The average AcChR distribution is of 8.000 molecules per µm^{2} but the AcChR are more concentrated at the top of the postsynaptic folds

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* In the innervated skeletal muscle, AcChRs are located almost exclusively in the postsynaptic membrane. But when the nerve innervating a muscle fiber is cut at a certain distance from the endplate, one observes a spreadout of the AcCh sensitivity over the whole muscle membrane (Thesleff, 1960) and the number of extrajunctional AcChRs increases 10-40 times (Hartzell and Fambrough, 1972, Almon and Appel, 1976).

(20-25.000 per μm^2, Barnard et al., 1975). Only a small fraction of the AcChRs are activated when a nerve impulse triggers the release of 3-5 x 10^6 AcCh molecules*. Thus, only a small proportion of the AcChRs present in the endplate need to be activated in order to depolarize the muscle membrane sufficiently to cause contraction of the cell. The excess AcChR is termed "safety margin But this does not mean that all the AcChRs in excess function as "spare receptors". As a matter of fact, blockade with α-Bgt of more than 25% of the total pool of receptors at the rat diaphragm endplate is sufficient to cause a reduction of the endplate potential (Barnard et al., 1975). The same authors showed that the sensitivity to applied AcCh is probably not determined by the tota number of AcChRs but by their local density.

The depolarization of the postsynaptic membrane after binding of AcCh to the AcChR is due to the opening of ionic channels (ionophores) to small ions like Na^+, K^+ and Ca^{++}. This is probably caused by a conformational change of the AcChR upon AcCh binding.* In 1967, Karlin suggested on the basis of the allosteric model of Monod et al. (1965) that the AcChR molecule exists in two conformations, one active (associated with increased ionic permeability {ionophore open}) and one inactive (ionophere shut). Recentl Grünhagen and Changeux (1976) showed, by using fluorescent probes,

* There is evidence that the loss of AcCh through lateral diffusion and AcChE hydrolysis prior to arrival at the AcChRs is considerable (Barnard, 1974). According to Katz and Miledi (1973), 66% of the AcCh molecules which reach AcChRs become reversibly bound. This ensures a highly efficient response per AcCh molecule.

**It is not yet known whether the AcChR itself is the molecule involved in the selective influx of small ions or whether there exists a molecule, the ionophore, separate from the AcChR, although closely associated with it. The discovery of the properties of histrionicotoxin (HTX) favors this latter hypothesis. This toxin, isolated from the skin of a Colombian frog (Daly et al., 1971), has been shown to bind to the postsynaptic skeletal muscle membrane and to block the ion conductance system without preventing AcCh binding (Dolly et al., 1977). The molecule binding HTX has been called the ion conductance modulator (ICM) (Albuquerque et al., 1973b). Very recently, Eldefrawi et al. (1977) showed that they could separate the AcChR from the HTX binding molecule after solubilization of *Torpedo* electric organ membranes with detergent. According to these results, the ICM is a molecule distinct from the AcChR but closely associated with it in the membrane. It may be the ionophore itself or a separate structure that links the ionophore to the AcChR.

that the AcChR in *Torpedo* membrane fragments undergoes conformational changes upon ligand binding. They propose a three-state model for the AcChR molecule which combines both the model for desensitization described by Katz and Thesleff (1957) and the model for allosteric transitions proposed by Monod et al. (1965). According to them, the AcChR exists in three structural states (R), (A) and (D) in reversible equilibria, each being able to bind a cholinergic effector (E). The (R), (A) and (D) states are related to the physiological "resting" (ionophore shut), "activated" (ionophore open) and "desensitized" (ionophore shut after prolonged application of agonist) states of the excitable membrane. Cholinergic antagonists stabilize the (R) or (D) state but not the (A) state. Blocking of the response to agonists therefore takes place via two different mechanisms: occupation of the receptor sites (R) in the resting state as (RE) or transformation of the receptor protein from the (R) state into the (DE) state upon (E) binding. The affinity of the receptor for cholinergic agonists is highest in the (D) state (Weber et al., 1975). The exposure to cholinergic agonists first causes a fast structural transition, the activation, associated with opening of the ionophore. This is followed by a slow and reversible change of structure, the desensitization, associated with a reduction of the permeability to cations.

Biochemical characteristics of AcChRs

Both electric organ (Changeux et al., 1975) and skeletal muscle AcChRs (Chiu et al., 1973) are integral membrane glycoproteins which can only be solubilized with detergent. From data obtained by gel filtration of these detergent-solubilized receptors, molecular weights of about 360.000 daltons were calculated after correction for detergent binding, considering that Triton X-100 contributes to 21% of the total mass of the AcChR-detergent complex (Meunier et al., 1972, Chiu et al., 1973, Edelstein et al., 1975).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions shows that AcChR consists of

several subunits: according to Claudio and Raftery (1977), *Torpedo* AcChR consists of four subunits with apparent molecular weights of 40-43K*, 48-52K, 59-60K and 64K. *Electrophorus electricus* AcChR consists of two or three subunits of about 40, 50 and 60K (for a review see Maelicke et al., 1977)** and rat skeletal muscle AcChR contains 4 subunits of 45, 51, 56 and 62K (Froehner et al., 1977a). Four subunits (44, 53, 65 and 72K) were also found in AcChR extracted from the muscle cell line BC_3H1 (Boulter and Patrick, 1977). It is widely admitted that the subunit of about 40K carries the AcCh binding site: both in *Electrophorus* (Karlin and Cowburn, 1973) and *Torpedo* AcChR (Weill et al., 1974) this subunit is labeled with the affinity alkylating agent (^{3}H)-p-(N-maleimido)-2-benzyltrimethylammonium (^{3}H-MBTA) (Karlin 1977b)***. It is still uncertain whether or not there are as many AcCh binding sites as neurotoxin binding sites per AcChR molecule (Fulpius, 1976). In *Torpedo* and eel AcChR, for instance, there are twice as many neurotoxin than MBTA binding sites whereas in rat skeletal muscle AcChR there is an equal number of both sites (Froehner et al., 1977b).

Recently, Sobel et al. (1977) showed that a neutral detergent extraction of highly purified *Torpedo* AcChR-rich membranes yielded two major components on SDS-PAGE. One component, with an apparent molecular weight of 40K, is labeled with ^{3}H-MBTA and presumably carries the AcCh binding site. The other component, with an apparent molecular weight of 43K, binds ^{3}H-histrionicotoxin in absence of detergents (Sobel et al., 1978). These authors suggest that the AcCh-receptor site and the site for HTX are carried by different entities (see also Eldefrawi et al., 1977).

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* K = kilodaltons = 1.000 daltons

** The discrepancy in the molecular weights of the subunits observed can be due to the techniques used (SDS-PAGE). It is known that SDS-PAGE is not very accurate for hydrophobic glycoproteins because comparable calibration standards are not available (Karlin 1977a).

***MBTA specifically alkylates a sulfhydryl group within the AcCh binding site of reduced AcChR (Karlin, 1977b).

The removal of the AcChR from its membrane environment with detergents might release a "membrane constraint" exerted by neighbouring membrane proteins and/or phospholipids which stabilizes one of the conformations of the AcChR molecule (see above). This would explain why the solubilization of AcChR with detergents alters the apparent binding affinity of the AcChR for cholinergic ligands. For example, the protection constant K_p* of carbamylcholine for *Torpedo* AcChR is of 4×10^{-8}M when the AcChR is membrane bound and of 5×10^{-5}M when the AcChR is solubilized. The K_p for d-TC are of 3×10^{-8}M and 5×10^{-6} respectively (for a review see Maelicke et al., 1977).

AcChRs from different electric fish have isoelectric points from 4.5 to 4.8 (Eldefrawi and Eldefrawi, 1977). Junctional AcChR from rat diaphragm muscle has an isoelectric point of 5.2 and extrajunctional AcChR of 5.5 (Brockes and Hall, 1975b).

Junctional and extrajunctional AcChR differ in several other characteristics: (i) their rate of turnover: the half-time of degradation of AcChR is of 6 days for junctional AcChR and of 18 to 20 hours for extrajunctional AcChR (Chang and Huang, 1975); (ii) their sensitivity to d-TC: the apparent dissociation constant is of 4×10^{-8}M for junctional AcChR and of 5.5×10^{-7}M for junctional AcChR (Brockes and Hall, 1975a); (iii) their antigenicity: serum from myasthenic patients inhibits ^{125}I-α-Bgt binding to rat extrajunctional and not to junctional AcChR (Almon and Appel, 1975); anti-*Torpedo* AcChR antibodies bind only to junctional AcChR from human and rat muscle section whereas myasthenic serum binds to both junctional and extrajunctional AcChR (Trotter et al., 1977).

Anti-AcChR antibodies

During evolution, living organisms have developed defense mechanisms against the intrusion of pathogens like bacteria, viruses and toxic polypeptides. These defense mechanisms are described

* The protection constant K_p is the concentration of ligand which inhibits the initial rate of toxin binding to the AcChR by 50%. This concentration approximates the apparent dissociation constant at equilibrium (K_D) for that ligand (Weber and Changeux, 1974).

under the name of immune response and the external agents are called antigens.

The most potent antigens (those which easily induce an immune response) are macromolecules like proteins and glycoproteins.

When an animal is immunized with a protein emulsified in Freund's adjuvant, several events are triggered (T and/or B lymphocyte activation {Ford 1973}). This leads to the synthesis of antibodies which will clear the organism of the antigen.

The antibody produced (mainly γ-immunoglobulin {IgG}), is a glycoprotein with a molecular weight of 150.000 daltons. It consists of 4 subunits, two heavy chains (50.000 daltons) and two light chains (20.000 daltons).

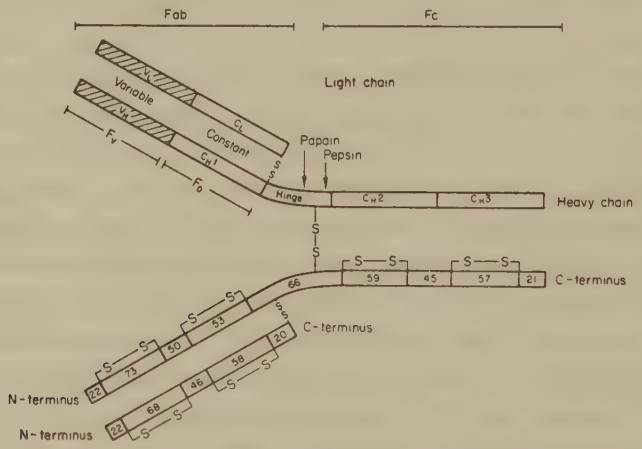

Fig. 1.1. A typical IgG molecule.
In the upper part of the figure, the names of the various parts of the molecule are given, while in the lower part, the number of amino acids in typical domains, and the arrangements of the intrachain disulphide bridges are shown. (Adapted from Milstein and Svasti, 1971.)

(taken from Hobart and McConnell, 1975)

The IgG molecule consists of constant (F_C, F_D) and variable (F_V) regions. Several amino acid residues in the variable region

form the site which binds to the antigen. There are two antibody combining sites per IgG molecule (the IgG is divalent)*.

A glycoprotein like the AcChR is a macromolecule which contains several distinct antigenic determinants. Thus, the immunized animal synthesizes immunoglobulins with various distinct specificities (different variable regions) capable of recognizing the different antigenic determinants on the antigen. The antibody population of an immunized animal is therefore always heterogeneous.

It is widely accepted that the body is generally unresponsive to its own constituents (self, Allison 1971). But autoimmune reactions (immune reaction against self) can be readily induced when normal animals are injected either with autologous (self) or heterologous (non-self) antigens emulsified in Freund's adjuvant (Clagett and Weigle, 1974). Anti-AcChR antibodies are usually produced by immunizing for instance rats or rabbits with detergent-solubilized AcChR from electric organs of fish (see foreword). The electric organ AcChR has a structure similar, but not identical to that of rat or rabbit AcChR (see above). Thus, some antigenic determinants of electric organ AcChR are identical to those of mammalian AcChR whereas others are not. The more identical antigenic determinants there are, the better the antibodies raised against electric organ AcChR will recognize the mammalian AcChR. This is termed antigenic cross-reactivity**. A rat immunized with eel AcChR, for instance, has a titer of antibodies against rat AcChR (autoantibodies) which is one hundredth of the titer against eel AcChR (RIA) (Lindstrom, 1976a). Thus, though the cross-reaction is small, it is possible that the anti-eel AcChR antibodies raised in the rat bind to the rat AcChR in situ and induce the neuromuscular blockade observed in EAMG***.

* If one digests the IgG with papain, one obtains monovalent F_{ab} fragments which still bind to the antigen.

** A review on cross-reactivity between *Torpedo*, eel, rat and frog AcChR is given by Lennon (1976).

***It might be that the antibodies damage the AcChR (see below) and the modified antigen could then trigger an intensified production of antibodies against self antigen (see Hall C.L. et al., 1977 for antibodies to tubular basement membrane). But according to Lindstrom et al. (1976c), the AcChR is not sufficiently altered after passive transfer of anti-AcChR antibodies to break down tolerance and trigger an autoimmune response.

When an antibody binds to an antigen present on a cell membrane, it can neutralize it in three different ways:

1) the antibody binds to the active site of the protein (substrat binding site of an enzyme or acetylcholine binding site of the AcChR) and suppresses its biological activity.

2) the first component of the complement system binds to the F_c portion of the antibody which is bound to the antigen. This initiates the activation of a series of components of the complement sequence (triggering of an enzyme cascade {McFarlan 1969}) which finally cause membrane lesions (Engel et al., 1977). The complement sequence can be inactivated by heating the serum for 30 minutes at 56°C.

3) the divalent antibodies cross-link the antigens on the cell surface (formation of a lattice) and induce a redistribution of the antigens (formation of patches). This leads to internalization (modulation) and then destruction of the antigen-antibody complexes (Taylor et al., 1971). Monovalent antibody F_{ab} fragments cannot induce any modulation. Modulation depends on the fluidity of the membrane. It does not take place at temperatures lower than 15°C. (Anwyl et al., 1977 and Bevan et al. 1977).

METHODS WHICH CAN BE USED TO ANALYSE THE EFFECT OF ANTI-AcChR ANTIBODIES ON THE AcChR

A) INTRODUCTION

Anti-AcChR antibodies raised in animals after immunization with AcChR isolated either from fish electric organs or from rat denervated skeletal muscle can be characterized by using different AcChR preparations. In all cases, the AcChR preparations are incubated in presence of either anti-AcChR serum or IgG purified from that serum. The effect of these antibodies on the AcChR is measured and compared with AcChR preparations incubated in presence of control serum by using the methods described below.

The following AcChR preparations are available:

1) detergent-solubilized AcChR (usually purified AcChR)
2) AcChR-rich membrane fragments
3) tissue sections from organs containing AcChR
4) intact cells of organs containing AcChR

With detergent-solubilized AcChR, antigenic sites usually hidden within the membrane are accessible to the antibodies (if they are not masked by detergent molecules). With AcChR-rich membrane fragments and on whole cells and organs, the AcChR is in its original membrane environment with only hydrophilic antigenic sites accessible to the antibodies.

These different AcChR preparations can be used to:

1) quantitate the anti-AcChR antibodies by measuring their binding to solubilized AcChR (radioimmunoassay)

2) to detect against which parts of the AcChR the anti-AcChR antibodies are directed (e.g. inhibition of toxin

binding to solubilized AcChR or AcChR-rich membrane fragments).

3) to analyse the physiological effect of the anti-AcChR antibodies on whole cells or organs (e.g. on the sensitivity to iontophoretically applied acetylcholine).

4) to measure the influence of the anti-AcChR antibodies on the metabolism of the AcChR (e.g. on the turnover-rate of the AcChR in muscle cells in culture).

B) LIST OF METHODS AVAILABLE

1) Detergent-solubilized AcChR

The AcChR is extracted from electric organs of fish or from denervated skeletal muscle with detergent and purified as referred to in paper (2).

a) Radioimmunoassay

The antigen used in this assay is a complex made of purified AcChR and radiolabeled neurotoxin. By incubating the anti-AcChR antibodies with the complex, only antibodies directed against other sites than the toxin binding site of the AcChR are measured (Patrick et al. 1973b, Lindstrom 1976d).

b) Inhibition of ^{125}I-α-T binding to the AcChR

In this assay one measures the antibodies which prevent toxin binding to the AcChR by incubating the AcChR in presence or in absence of anti-AcChR serum and ^{125}I-α-T. This is a way of detecting antibodies directed against the toxin binding site of the AcChR (Almon et al. 1974).

2) AcChR-rich membrane fragments

AcChR-rich membrane fragments are prepared from electric organs of *Torpedo* (see next chapter) or *Electrophorus electricus* (Teichberg and Changeux 1977).

a) Inhibition of ^{125}I-α-T binding

Membrane fragments are incubated in presence or absence of anti-AcChR serum and ^{125}I-α-T (see below).

b) Inhibition of binding of labeled anti-AcChR antibodies

Membrane fragments are incubated in presence of neurotoxin or cholinergic pharmalogical agents and ^{125}I labeled anti-AcChR antibodies (see below).

3) AcChR on tissue sections

10 μ sections from frozen muscle preparations known to contain neuromuscular junctions by histochemical staining of acetylcholinesterase are made.

a) Inhibition of toxin binding

Autoradiographic measurement of ^{125}I-α-Bgt binding to muscle sections are made in presence or absence of myasthenic serum (Bender et al. 1975).

b) Binding of anti-AcChR antibodies coupled with ferritin to eel or *Torpedo electroplax*

(Karlin et al. 1978)

4) AcChR in intact cells or whole organs

i) Electric organ of fish

Single cells (electrocytes) from the electric organ of *Electrophorus electricus* are dissected out and incubated in Ringer's solution containing anti-AcChR antibodies or control antibodies.

a) Inhibition of toxin binding

The number of sites per electrocyte labeled with ^{125}I-α-T are measured after incubation in presence or absence of anti-AcChR serum (Lindstrom 1976d).

b) Response to bath-applied carbachol

A microelectrode is implanted into a single electrocyte. Depolarization induced by carbachol is measured in presence and absence of anti-AcChR antibodies (Patrick et al. 1973b, Sugiyama et al. 1973).

ii) Skeletal muscle cells in culture

Cultures of rat skeletal muscle, human muscle and mouse muscle cell lines are made.

a) Inhibition of toxin binding

^{125}I-α-Bgt binding is measured after incubation of the cells in either anti-AcChR or control serum (Heinemann et al. 1977).

b) Degradation rate and turnover of AcChR

The turnover of AcChR is determined by measuring the radioactivity released into the medium after labeling of the cells with ^{125}I-α-Bgt and subsequent incubation with antiserum (Heinemann et al. 1977, Appel et al. 1977, Kao and Drachman 1977).

c) Sensitivity to iontophoretically applied acetylcholine

Intracellular recordings of depolarizations in response to acetylcholine are made with microelectrophysiological techniques after incubation with or without anti-AcChR serum (Anwyl et al. 1977, Bevan et al. 1977).

iii) Whole skeletal muscle

a) Inhibition of toxin binding

Binding of labeled neurotoxin to the neuromuscular region of mouse diaphragm is measured after incubation in presence or absence of anti-AcChR serum (Keesey et al. 1976, paper (2)).

b) Sensitivity to iontophoretically applied acetylcholine

Depolarizations in response to bath-applied acetylcholine are measured in innervated and denervated muscle fibers after incubation with or without anti-AcChR serum (Albuquerque et al. 1976, Bevan et al. 1976).

c) Measurement of MEPP and EPP on a nerve-muscle preparation

Electrophysiological recordings are made in presence or absence of anti-AcChR antibodies with microelectrodes implanted into muscle cells (sciatic nerve-sartorius muscle preparation) (Albuquerque et al. 1976, Green et al. 1975)

d) Measurement of amplitude of contraction of nerve-diaphragm preparation

The amplitude of contraction of a diaphragm muscle is measured during electrical stimulation of the nerve and in presence or absence of anti-AcChR serum (Berti et al. 1976, see methods).

iv) Whole animal

Anti-AcChR antibodies are injected intra-venously into an animal. Measurements of their effects on the neuromuscular junction are made by electromyography,

recording of EPP and MEPP, quantitation of total amount of AcChR and electron microscopy (Toyka et al. 1975, Lindstrom et al. 1976c).

v) AcChR in myasthenic patients and animals with EAMG

Anti-AcChR antibodies can be localized in the post-synaptic membrane of neuromuscular junctions by electron microscopy after labeling with peroxidase-conjugated protein A (Engel et al. 1977).

METHODS USED IN THIS WORK TO ANALYSE THE EFFECT OF ANTI-AcChR ANTIBODIES ON THE AcChR

Three different kinds of AcChR preparations were used in this work to characterize the anti-AcChR antibodies:

1) detergent-solubilized, purified Torpedo AcChR
2) AcChR-rich *Torpedo* membrane fragments
3) AcChR in whole mouse diaphragm

1) SOLUBILIZED AcChR

A) Radioimmunoassay

AcChR from frozen electric organs of *Torpedo marmorata* was purified by solubilization in 1% Triton X-100 and affinity chromatography on α-T-Sepharose as described in paper (2). α-Bgt was purified according to the method of Lee et al. (1972a) and α-T according to the method of Cooper and Reich (1972). They were both iodinated to specific activities of 10-50 Ci/mmol using the method described by Devreotes and Fambrough (1975). The preparation of the complex made of purified AcChR and ^{125}I labeled α-Bgt, the radioimmunoassay used to quantitate anti-AcChR antibodies and the immunization of rabbits with purified *Torpedo* AcChR (200-300 μgr per injection) are described in paper (2).

B) Inhibition of ^{125}I-α-T binding

To 0.2 ml of buffer A (0.05M Tris-HCl pH 7.4, 0.1M NaCl, 0.1% Triton) the following solutions were added:

a) 10 μl of buffer B (0.05M Tris-HCl pH 7.4, 0.1M NaCl, 1% Triton) containing 1.5 pmoles of *Torpedo* AcChR

b) 20 µl of phosphate buffered saline (PBS) containing varying amounts of anti-*Torpedo* AcChR antibodies (2 to 200 µgr of purified IgG completed to 400 µgr with control anti-phage T_4 IgG) or 400 µgr control IgG alone.

c) 20 µl of PBS containing 0.2, 0.35, 1, 2.5, 4.5 and 13 pmoles of ^{125}I-α-T.

The samples were incubated for 15 hours at room temperature. Free ^{125}I-α-T was separated from the ^{125}I-α-T-AcChR complex by gel filtration on plastic columns (0.8 x 28 cm) containing 10 ml Ultrogel AcA 44 (LKB) equilibrated in buffer A. The radioactivity of the different fractions was measured in an Autogamma Scintillation Counter (Beckman).

The percent inhibition of toxin binding due to the anti-AcChR antibodies was measured by substracting the amount of ^{125}I-α-T bound to the AcChR in the presence of anti-AcChR antibodies (X) from the amount bound in their absence (Y). This quantity (Y-X) was then divided by Y to give the percentage of inhibition of toxin binding. For all quantities of anti-AcChR antibodies studied the inhibition was maximal for amounts of ^{125}I-α-T equal to the amount of AcChR, that is 1-2 pmoles of ^{125}I-α-T. In further studies we therefore always used equal amounts of AcChR and ^{125}I-α-T and incubated them in presence of increasing quantities of anti-AcChR antibodies or anti-AcChR serum. These experiments are described in paper (2).

) AcChR-RICH MEMBRANE FRAGMENTS

A) Preparation of membrane fragments

Receptor-rich membrane fragments were prepared from 50-150 gr of frozen *Torpedo marmorata* electric tissue by

homogenization in a Virtis apparatus as described by Popot et al. (1976). The following modifications were made: 6 x 34 ml of the supernatant from a 10 minute centrifugation at 5.000 g (6.500 rpm in a SS-34 rotor of a R5 Sorvall centrifuge) filtered through cheese cloth were layered on 6 ml sucrose 1.2 M in 6 cellulose nitrate tubes (9 x 2.5 cm, Beckman) and centrifuged at 4°C for 90 minutes at 64.000 g (24.000 rpm in the SW27 rotor of a Beckman L2 65B centrifuge). The layer of membrane fragments on top of the 1.2 M sucrose was collected and diluted to a total volume of about 30 ml with H_2O containing 0.02% NaN_3. Protein was assayed by the method of Lowry et al. (1951) using bovine serum albumine as standard.

The number of α-Bgt binding sites in these fragments was measured as follows: 5, 10, 15 and 20 µl of a solution diluted 1:10 containing the membrane fragments, 10 pmoles of ^{125}I-α-Bgt (in 10 µl) and 100 µl of rabbit anti-T_4 control serum were added to 0.2 ml PBS in 4 polycarbonate thick-wall tubes (1.6 x 7.6 cm, Beckman). After a 60 minute incubation at room temperature, the tubes were centrifuged for 30 minutes at 66.000 g (40.000 rpm in the Ti 50 rotor of a Beckman L2 65B centrifuge). The supernatant was removed, the pellet resuspended in 3 ml PBS and the tubes centrifuged again 30 minutes at 66.000 g. After removal of this second supernatant, the radioactivity of the pellets was determined. The suspension of membrane fragments were stored at 4°C in presence of 0.02% NaN_3, 100 µg/ml streptomycine and 10^4 units of penicillin G, and used for several weeks.

Inhibition of ^{125}I-α-T binding

Five µl of a suspension of membrane fragments containing 2 pmoles of AcChR and increasing amounts of anti-AcChR

serum (1, 2, 5, 10 and 20 µl, completed to a total of 100 µl with anti-T_4 control serum) were added to 0.2 ml PBS. Two pmoles of ^{125}I-α-T were added after 5 minutes and the incubation was pursued for one hour at room temperature. The samples were then centrifuged 2 times 30 minutes at 66.000 g and the radioactivity of the pellets determined as described above. The percent inhibition of toxin binding was measured as described for solubilized AcChR.

The inhibition observed with increasing amounts of a given serum was compared to the inhibition obtained on the same fragments after incubation in buffer B (containing 1% Triton) instead of PBS. In this case, the amount of inhibition was measured after gel filtration on Ultrogel AcA 44 as described for solubilized AcChR. The percentages of inhibition of toxin binding per µl serum (derived from curves as those shown in Fig. 1, paper (2)) obtained on intact membrane fragments were compared to those obtained on fragments solubilized in detergent.

C) Inhibition of ^{125}I-anti-AcChR binding

Purified rabbit anti-*Torpedo* AcChR immunoglobulins and sheep anti-rabbit immunoglobulins (SARIG) were iodinated to specific activities of 40 Ci/mmole and 55 Ci/mmole respectively with the ICl method described for anti-toxin antibodies in paper (1).

To 0.2 ml PBS, 5 µl of a suspension of membrane fragments containing 2 pmoles of AcChR, 100 µl of anti-T_4 serum and 10 µl PBS containing 1.7, 3.7, 7, 12 and 16 pmoles of either ^{125}I-anti-AcChR or ^{125}I-SARIG were added. In parallel series, either 2 µl of α-Bgt (final concentration = 3×10^{-6}M), 30 µl of hexamethonium (10^{-2}M) or 30 µl carbamylcholine (10^{-2}M) were added 5 minutes before the

addition of ^{125}I-anti-AcChR or ^{125}I-SARIG. After a one hour incubation at room temperature, the tubes were centrifuged 2 times 30 minutes at 66.000 g and the radioactivity of the pellets measured as described above.

D) Desorption of ^{125}I-anti-AcChR antibodies

To 0.2 ml PBS, 100 µl of a suspension of membrane fragments containing 40 pmoles of AcChR (180 µgr protein), 10 µl PBS containing 120 pmoles of ^{125}I-anti-AcChR (18 µgr protein) and 100 µl of anti-T_4 serum were added and incubated for one hour at room temperature. At the end of the incubation the fragments were diluted to a final volume of 25 ml with PBS and layered on top of a discontinuous sucrose gradient consisting of 2 ml fractions of a 1.5, 1.4, 1.3, 1.2, 1.1, 1.0 and 0.8M sucrose solution in a cellulose nitrate tube. The tube was centrifuged for 4 hours at 64.000 g in a SW 27 swinging rotor. Forty fractions of approximately 1 ml were collected after perforation of the bottom of the tubes and their radioactivity measured in a gamma counter. The fractions containing ^{125}I-anti-AcChR bound to the membrane fragments were pooled and devided into 4 equal volumes (about 2 ml) containing each about 1 pmole of ^{125}I-anti-AcChR.

Several preparations like these were incubated for one hour at room temperature with either NaCl (final concentration in 4ml = 2M) or $MgCl_2$ (2M), or for 6 hours at room temperature with α-Bgt or α-T (final concentration = 5-10 x 10^{-6}M), NaCl (10^{-1}M), carbamylcholine (10^{-1}M) or d-TC (10^{-3}M). After incubation, the tubes were centrifuged for 2 hours at 66.000 g. The amount of radioactivity present in the supernatants was determined.

E) Adsorption of unlabeled anti-AcChR antibodies on membrane fragments

To 30 ml of a suspension of membrane fragments containing 66 mg protein and 6 nmoles ^{125}I-α-Bgt binding activity, 100 µl of a solution containing 2.3 mg purified anti-AcChR antibodies (IgG) were added as well as BSA (final concentration 5 mg/ml). After a one hour incubation at room temperature under constant gentle stirring, the suspension was layered on 6 ml of a 1.2 M sucrose solution and centrifuged for 90 minutes at 64.000 g in a SW 27 rotor. The supernatant (about 30 ml) containing the antibodies not adsorbed on the fragments was loaded on a column (5 x 1 cm) containing 1 ml protein A covalently coupled to Sepharose (Pharmacia). The loading was performed at room temperature and at a flow-rate of 9 ml per hour using a peristaltic pump (Pharmacia). The solution was then recycled over-night at the same flow-rate. After washing the column with 50 ml PBS, the immunoglobulins adsorbed to protein A were desorbed with 5 ml of glycine-HCl 0.1M pH 2.6 and immediately dialyzed over-night at 4°C against 2l of PBS (Union Carbide dialysis tubing, Serva). The protein concentration of the desorbed material was determined by the method of Lowry et al. (1951).

The anti-AcChR antibody titer was measured by RIA as described in paper (2) by taking 1 to 5 µg of non-adsorbed antibodies. 20-100 µg of these same anti-AcChR antibodies are also used to inhibit ^{125}I-anti-AcChR binding to membrane fragments as described above.

3) AcChR IN WHOLE ORGANS

A) Inhibition of ^{125}I-α-T binding to mouse diaphragm

Diaphragms were dissected out from C3H mice and incubated with decomplemented anti-AcChR serum and ^{125}I-α-T as

described in paper (2).

B) Inhibition of contraction of a nerve diaphragm preparation

Phrenic nerve-diaphragm preparations from adult albino rats (Wistar) were dissected out and suspended in a vessel containing 15 ml of a Krebs solution prepared as described by Laity et al. (1969) (= maximal capacity of the vessel). The solution was kept at 37°C and bubbled with 95% oxygen and 5% carbon dioxide. The phrenic nerve was stimulated by means of a Grass stimulator with rectangular pulses of 50 V amplitude and 1.4 msec. duration at a frequency of 0.2 Hz. D-TC (3×10^{-6}M), α-T (4×10^{-7}M) and 1 to 15 ml anti-AcChR serum (serum raised either against purified *Torpedo* AcChR (rabbit no 3) or serum raised against AcChR-rich *Torpedo* membrane fragments as described by Berti et al. (1976)(rabbit no 63) were added to the medium (the amount of Krebs solution was decreased correspondingly). The muscle contractions were recorded with an isometric transducer.

RESULTS

1) CHARACTERIZATION OF ANTI-AcChR ANTIBODIES USING SOLUBILIZED AcChR

A) Radioimmunoassay

(quantitation of anti-AcChR antibodies directed against sites other than the toxin binding site of the AcChR)

Anti-*Torpedo* AcChR antibody titers ranging from 1 to 7 nmoles ^{125}I-α-Bgt-AcChR complex precipitated per ml serum were measured in 7 different rabbits at the time of appearance of paralysis (Table I). As shown by other authors (Lindstrom et al., 1976a, Lambert et al., 1976) and in paper (2), there is no correlation between the amount of the anti-AcChR detected by this method and the clinical status of the animal.

B) Inhibition of ^{125}I-α-T binding

(detection of anti-AcChR antibodies directed against the toxin binding site of the AcChR)

Fig. 1 shows the amount of ^{125}I-α-T-AcChR complex formed with increasing concentrations of ^{125}I-α-T in presence of control IgG or in presence of two concentrations of anti-AcChR IgG from rabbit no 3 (22.7 and 113 µg). Anti-AcChR antibodies from rabbit no 3 inhibited toxin binding to the AcChR by up to 60% for concentrations of ^{125}I-α-T equal to the concentration of AcChR ($1-2 \times 10^{-9}$M). Anti-AcChR antibodies from rabbit no 1 gave a maximal inhibition of 77%.

When the data of Fig. 1 are expressed in a plot 1/RT versus 1/free toxin (Fig. 2), all the curves have the same intersection point on the ordinate. This intersection

TABLE I

AMOUNT OF ^{125}I-α-BGT - AcChR COMPLEX PRECIPITATED WITH SERA FROM RABBITS IMMUNIZED WITH *TORPEDO* AcChR (RIA)

(The sera are taken at the time of appearance of signs of paralysis)

rabbit	nmoles complex precipitated per ml serum	*Torpedo* AcChR
1	5	detergent-solubilized, purified AcChR
3	1.47	"
4	0.78	"
5	7	"
7	1.8	"
8	1.3	"
9	5.44	"
24	0.025	solubilized AcChR boiled for 10 minutes at 100°C
63	0.526	AcChR-rich membrane fragments

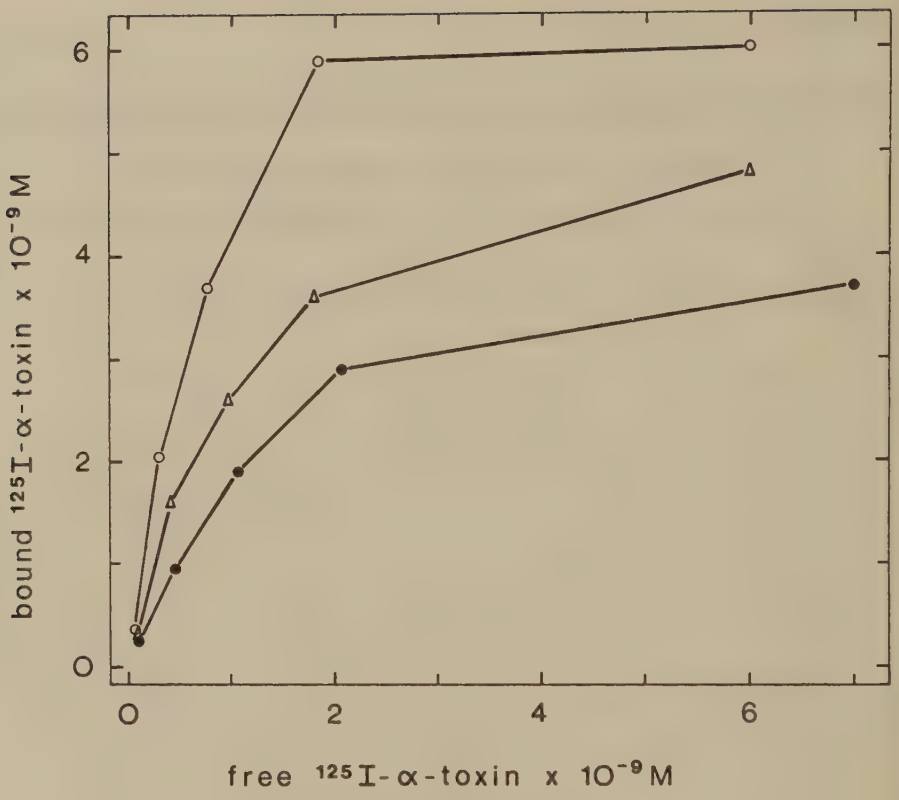

Fig. 1 Amount of ^{125}I-α-T-AcChR complex formed (^{125}I-α-T bound) with increasing concentrations of ^{125}I-α-T in presence of 400 µg control IgG (o———o), 22.7 µg anti-AcChR IgG (Δ———Δ) and 113 µg anti-AcChR IgG (●———●)

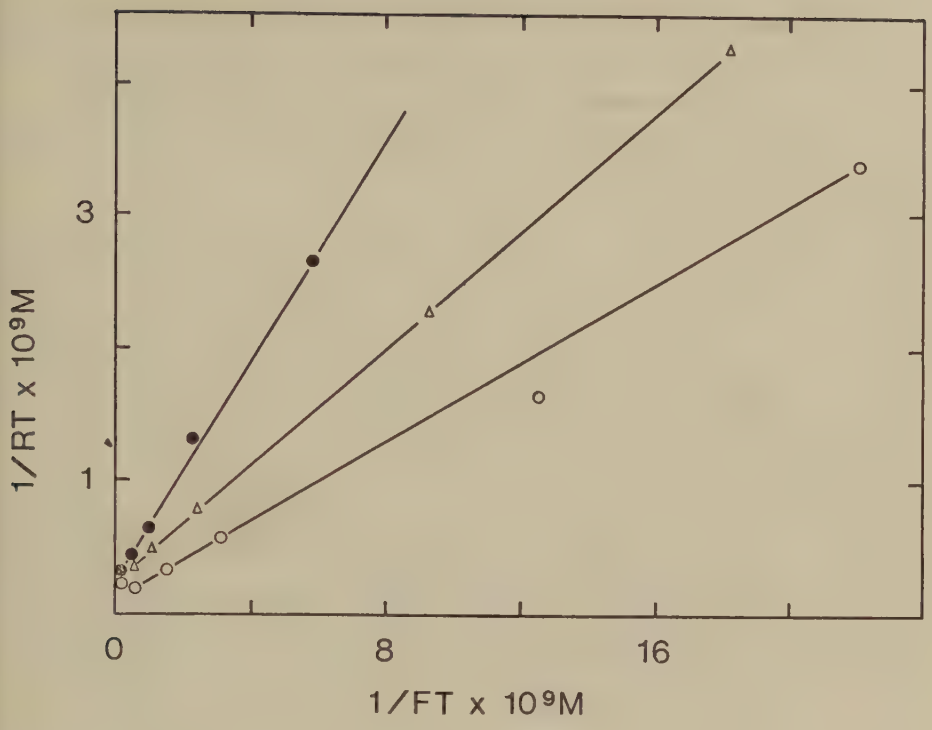

Fig. 2 Double-reciprocal plot of the curves shown in Fig. 1. RT = ^{125}I-α-T-AcChR complex; FT = free ^{125}I-α-T

point corresponds to 1.2 pmoles of ^{125}I-α-T-AcChR complex which is the amount of AcChR added initially to the incubation medium. Thus, when a sufficient amount of ^{125}I-α-T is present, anti-AcChR antibodies do not prevent toxin binding to the AcChR.

Fig. 1 paper (2) shows the percentage of inhibition of toxin binding produced in presence of increasing amounts of serum from rabbit no 5. The relationship obtained shows that, above a certain concentration of antibodies, the amount of inhibition does not increase linearly with the amount of antiserum added.

Inhibition of toxin binding was also measured with the serum of a rabbit immunized with *Torpedo* AcChR boiled for 10 minutes at 100°C (rabbit no 24)*. Using as much as 300 µl of this serum (which precipitate 7.4pmoles of ^{125}I-α-Bgt-AcChR complex, see table 1), no inhibition of ^{125}I-α-T binding to *Torpedo* AcChR was observed. By comparison, 5 µl of a serum raised against native *Torpedo* AcChR (rabbit no 4) and precipitating 6.5 pmoles of ^{125}I-α-Bgt-AcChR complex by RIA, inhibited binding of toxin by 54%.

2) CHARACTERIZATION OF ANTI-AcChR ANTIBODIES USING AcChR-RICH MEMBRANE FRAGMENTS

A) Preparation of the fragments

50-150 gr wet tissue of *Torpedo* electric organ gave fragments containing 70-200 mg protein and 4-10 nmoles α-Bgt

* Footnote: This serum against denatured AcChR still recognizes native AcChR although to a lesser extent (a maximal titer of 24.6 pmoles native AcChR precipitated per ml serum was reached in this rabbit). We have no other way of quantitating the amount of antibodies directed against the denatured AcChR since denatured AcChR does not bind toxin any more. The rabbit immunized with denatured (boiled) AcChR did not become paralysed.

binding capacity. Thus, one gramm wet tissue yielded membrane fragments containing about one mg protein and binding 50-100 pmoles ^{125}I-α-Bgt. These suspensions could be stored for a few months at 4°C in presence of antibiotics as described under methods. One preparation, for example, lost only 30% of its α-Bgt binding capacity after a 4 month's storage at 4°C.

B) Inhibition of ^{125}I-α-T binding

Fig. 3 shows the percentages of inhibition of toxin binding produced on intact AcChR-rich membrane fragments as compared to those obtained on the same fragments solubilized with detergent. For serum no 5 taken when paralysis occurred, the inhibitions were 3.5% and 38% per µl serum respectively. Thus, this serum is 11 times less effective in inhibiting toxin binding to the AcChR when the receptor is in its membrane environment than when it is solubilized and surrounded by detergent.

C) Inhibition of ^{125}I-anti-AcChR binding

The amount of AcChR-rich membrane fragments needed to adsorb the anti-AcChR present in 60 pmoles ^{125}I-IgG purified from anti-AcChR serum no 3 (3% of the total ^{125}I-IgG) corresponds to 20 pmoles of ^{125}I-α-Bgt binding activity. FIg. 4 shows the amount of ^{125}I-anti-AcChR antibodies bound to the membrane fragments in presence of an excess of α-Bgt (4×10^{-6}M) or in its absence. In these conditions, α-Bgt inhibits binding by a maximum of 15% (results from 4 experiments). When more than 30 pmoles of ^{125}I-anti-AcChR were added, no inhibition of their binding to the fragments was observed with α-Bgt. When similar experiments were made in presence of hexamethonium (10^{-2}M) or carbamylcholine (10^{-2}M), no inhibition of ^{125}I-anti-AcChR was observed.

Two controls were made to test whether or not the binding of ^{125}I-anti-AcChR antibodies to the fragments was spe-

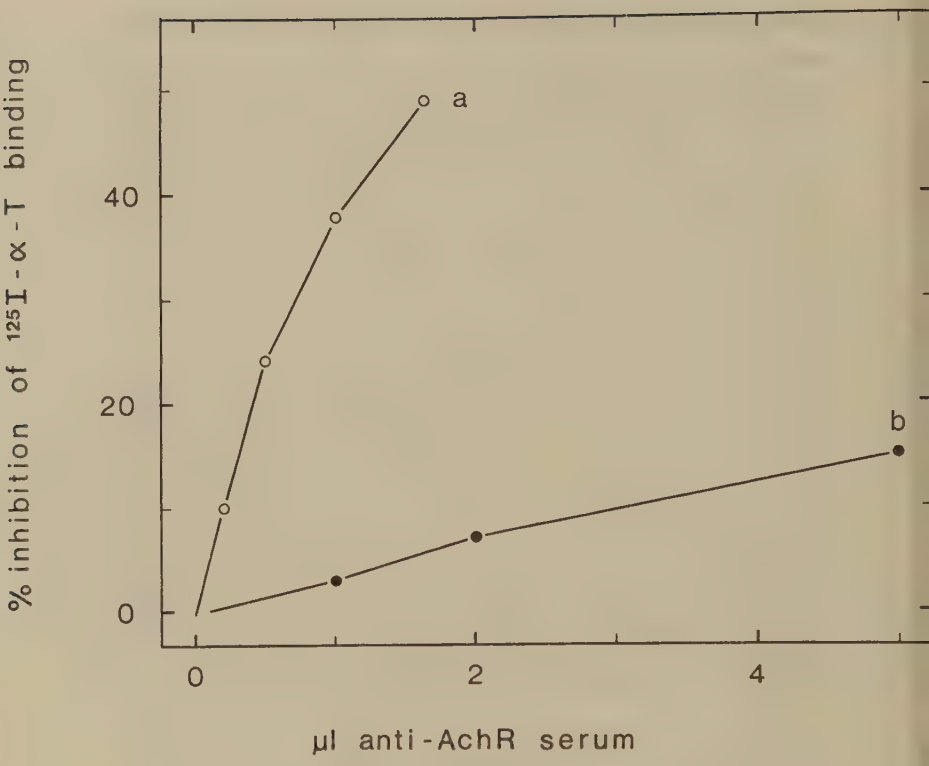

Fig. 3 Percentages of inhibition of ^{125}I-α-T binding produced with increasing quantities of serum from rabbit No 5 on intact AcChR-rich membrane fragments (b) and on the same fragments solubilized with detergent (a)

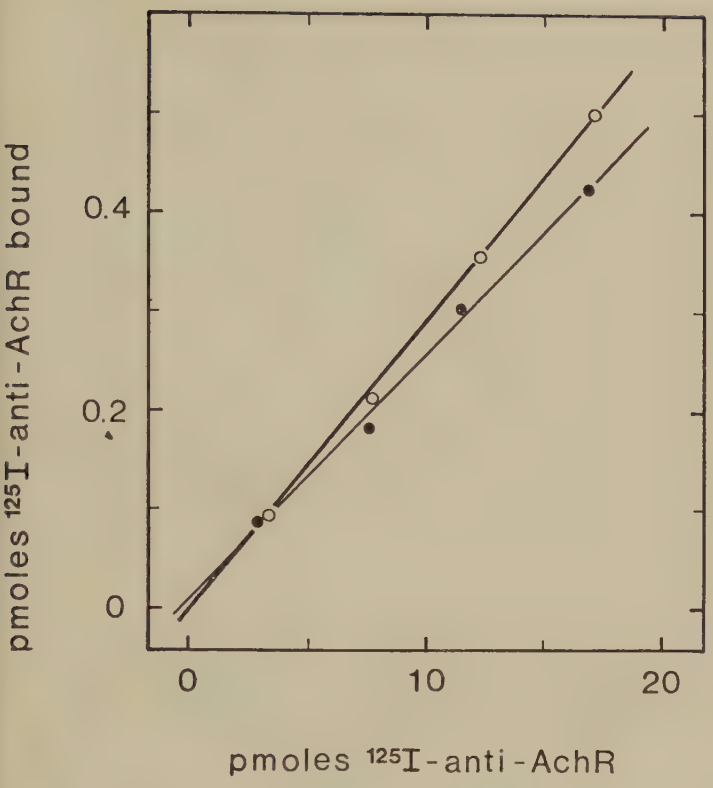

Fig. 4 Amount of ^{125}I-anti-AcChR antibodies bound to AcChR-rich membrane fragments in presence of 4 x 10^{-6} M α-Bgt (●——●) and in its absence (o——o)

cific:

a) 5 µl of a suspension of membrane fragments containing 2 pmoles of AcChR were incubated with 12 pmoles of ^{125}I-IgG purified from anti-AcChR serum no 3 and increasing amounts of non-labeled anti-AcChR antibodies from the same rabbit (1 to 400 pmoles) (Fig. 5). 400 pmoles of these anti-AcChR antibodies inhibited ^{125}I-anti-AcChR binding by a maximum of 70%. From Fig. 5, showing the percentage of ^{125}I-anti-AcChR bound in function of the amount of unlabeled anti-AcChR added to the medium, one can see that about 100 pmoles anti-AcChR antibodies are needed to inhibit by 50% the binding of 12 pmoles of ^{125}I-anti-AcChR antibodies.

b) The membrane fragments were also incubated with increasing amounts of ^{125}I-SARIG in presence and absence of α-Bgt as described under methods. In this case, the amount of radioactivity bound to the fragments is equal in each tube (3, 4, 7 and 12% of the total counts) and α-Bgt (4×10^{-6}M) does not prevent this nonspecific binding.

D) Desorption of ^{125}I-anti-AcChR antibodies

Fig. 6 shows the amount of radioactivity released from fragments preincubated with ^{125}I-anti-AcChR in presence of several cholinergic agonists and antagonists: carbamylcholine (10^{-1}M) and d-TC (10^{-3}M) do not desorb any ^{125}I-anti-AcChR antibodies whereas hexamethonium (10^{-1}M) desorb 4% of the counts, α-T (5×10^{-6}M) 3% and α-Bgt (5×10^{-6}M) 7%.

These percentages are averages from 3 to 4 experiments. 20% of the radioactivity remain bound to the fragments after treatment with $MgCl_2$ (2M). Treatment with NaCl (2M) shows that 50% of the counts are non-specifically bound to the fragments.

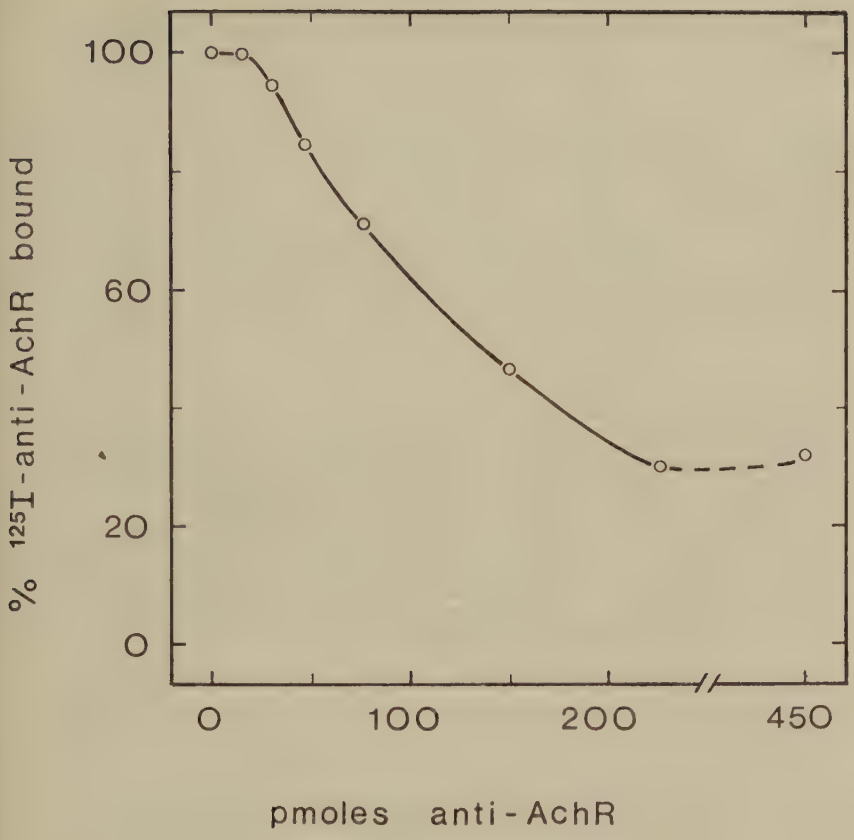

Fig. 5 Percentage of ^{125}I-anti-AcChR antibodies bound to AcChR-rich membrane fragments in presence of increasing concentrations of anti-AcChR antibodies

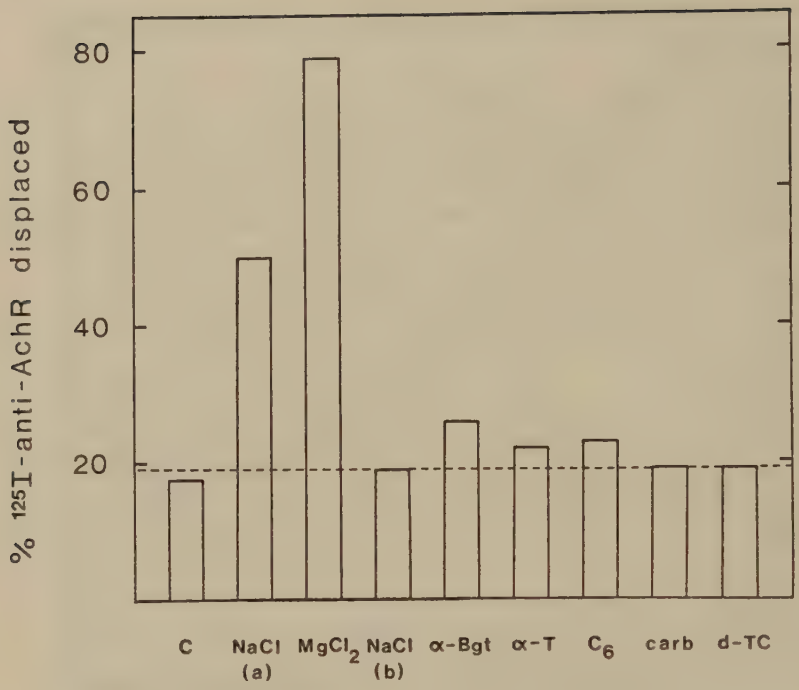

Fig. 6 Percentage ^{125}I-anti-AcChR antibodies released from AcChR-rich membrane fragments in presence of several cholinergic agonists and antagonists: control (C), 2M NaCl (a), 2M $MgCl_2$, 10^{-1}M NaCl (b), 5 x 10^{-6}M α-Bgt, 5 x 10^{-6}M α-T, 10^{-1}M hexamethonium, 10^{-1}M carbachol and 10^{-3}M d-TC

E) Adsorption of unlabeled anti-AcChR antibodies on membrane fragments

1.14 mg of anti-AcChR antibodies were adsorbed on membrane fragments containing 6 nmoles ^{125}I-α-Bgt binding sites. Before adsorption, 23 µg of these antibodies inhibited ^{125}I-anti-AcChR binding by 60%. After desorption, up to 120 µg of the anti-AcChR present in the supernatant did not inhibit ^{125}I-anti-AcChR to the fragments.

The same antibodies tested by RIA gave the following results (Fig. 7): 2.27 mg (A) or 1.14 mg (B) of the anti-AcChR antibodies were each adsorbed on the same amount of membrane fragments (6nmoles ^{125}I-α-Bgt binding capacity). Fig. 7 shows that the anti-AcChR of supernatant A have lost 55% of their activity after adsorption and those from supernatant B 96% (as compared to curve C). When we treated the fragments with 1M NaCl after adsorption of 1.14 mg antibodies (to desorb the antibodies nonspecifically bound to the fragments), the antibodies in the supernatant had still lost 90% of their activity. Experiments performed in this lab showed that when 1 to 2 mg of anti-AcChR antibodies were adsorbed repeatedly (4-5 times) with equal amounts of fresh membrane fragments, they progressively lost all their activity when measured by RIA. To decrease the amount of antibodies nonspecifically adsorbed to the fragments we have also pre-treated the fragments with 120 mg control IgG (anti-α-T antibodies) before using them to adsorb the anti-AcChR antibodies. Even then, the activity of the anti-AcChR antibodies in the supernatant had decreased by 80%.

) CHARACTERIZATION OF ANTI-AcChR ANTIBODIES USING AcChR IN WHOLE ORGANS

A) Inhibition of ^{125}I-α-T binding to mouse diaphragm

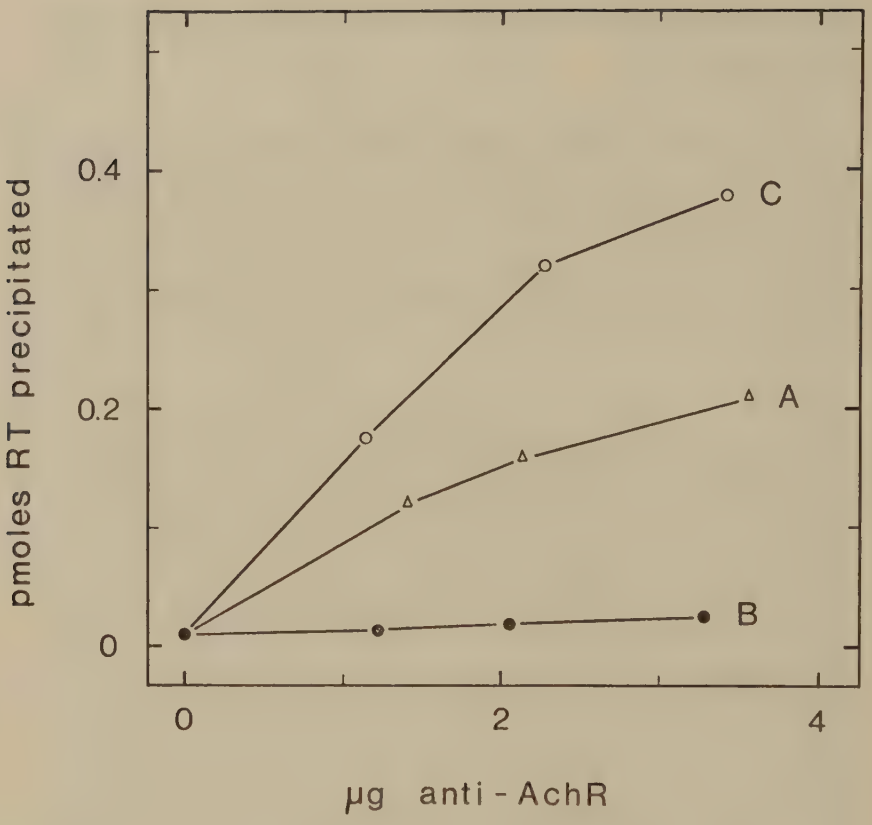

Fig. 7 Radioimmunoassay of anti-AcChR antibodies: amount of antibodies before adsorption on membrane fragments (C), amount of antibodies present in the supernatant after adsorption of 2.27 mg (A) and 1.14 mg (B) antibodies on membrane fragments. RT = ^{125}I-α-Bgt-AcChR complex

Results of the experiments done on mouse diaphragm are given in paper (2).

B) Inhibition of contraction on a nerve-diaphragm preparation

When a nerve-diaphragm preparation was incubated in presence of d-TC (3×10^{-6}M) the amplitude of contraction progressively decreased to 40% of maximum after 10 minutes. When the preparation was incubated in α-T with a final concentration of either 4×10^{-8}M or 4×10^{-7}M, the amplitude of contraction decreased by 50% after 45 minutes and 12 minutes respectively. After incubations of up to 120 minutes with 15 ml of antiserum against purified AcChR from rabbit no 3 (see Table I) or with 15 ml antiserum against AcChR-rich membrane fragments from rabbit no 63, no decrease in the amplitude of contraction could be measured.

paper (1)

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Accessibility to antibodies of acetylcholine receptors in the neuromuscular junction*

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SUMMARY

Antibodies to the acetylcholine receptor are present in the serum of myasthenic patients but one does not know if, *in vivo* and *in situ*, they can penetrate the intact neuromuscular junction and block directly the receptor.

The present experiments demonstrate that molecules the size of antibodies can reach acetylcholine receptor *in situ*. The mouse diaphragm with its intact neuromuscular junction was used as a source of acetylcholine receptor. The receptor was revealed either directly by iodinated α-bungarotoxin covalently coupled to IgG or indirectly, once labelled with cobra toxin, by iodinated anti-cobra toxin antibodies.

INTRODUCTION

Myasthenia gravis (MG) is a relatively rare human disease manifested by muscular weakness and fatigability. The clinical symptomatology has been attributed to a defective transmission at the neuromuscular junction. Yet the exact site of the defect is still debated. The involvement of the immune system in this disease is widely admitted nowadays.

In 1960, J. A. Simpson suggested that 'MG is an auto-immune response of muscle in which an antibody to end-plate protein may be formed. This would have the properties of an acetylcholine competitive blocking substance.' In recent years important progress has been made not only towards a better characterization of the two basic concepts expressed in Simpson's hypothesis (the acetylcholine receptor (AChR) and the antibodies directed against the receptor (anti-AChR)) but also towards a better understanding of their possible role in the pathogeny of the disease. Many groups have purified AChR from animal and even from human sources (for a review see Karlin, 1974). This molecule has been characterized *in vitro* (Karlsson, Heilbronn & Widlund, 1972; Klett *et al.*, 1973; Eldefrawi & Eldefrawi, 1973; Biesecker, 1973; Schmidt & Raftery, 1973; Meunier *et al.*, 1974; Chang, 1974) and identified *in situ* with α-bungarotoxin (αBgt) (Porter *et al.*, 1973; Fertuck & Salpeter, 1974; Daniels & Vogel, 1975). In neuromuscular junctions of myasthenic patients it has been shown that the number of receptors available was reduced (Fambrough, Drachman & Satyamurti, 1973). Anti-AChR have been raised in rabbits against electric eel AChR (Patrick & Lindstrom, 1973; Sugiyama *et al.*, 1973; Heilbronn *et al.*, 1975; Aharonov *et al.*, 1975). These animals seem to develop an autoimmune reaction whose symptoms resemble the muscular weakness found in MG. Recently, anti-AChR have also been found in the sera of myasthenic patients and shown to block the binding of αBgt to human AChR (Bender *et al.*, 1975). However, these results have been obtained only on tissue sections. Since, *in vivo*, anti-AChR should first cross the capillary wall, then penetrate the narrow synaptic cleft before reaching AChR

* Part of this study was presented at the 5th Conference on Myasthenia Gravis in New York, May 1975.

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in situ, one could wonder whether, in physiological conditions, molecules the size of immunoglobulins have such an easy access to the AChR molecules present in the neuromuscular junctions.

Using the mouse diaphragm with its intact neuromuscular junction as a source of AChR, we have tried to clarify this point and have obtained the following results: αBgt covalently coupled to IgG prevents subsequent labelling of the nerve terminals with iodinated αBgt; iodinated αBgt covalently coupled to IgG is detected in the neuromuscular junction; cobra toxin linked to AChR in the neuromuscular junction is subsequently revealed by iodinated anti-cobra toxin immunoglobulins. These results clearly show that molecules the size of antibodies can enter the synaptic cleft.

MATERIALS AND METHODS

Covalent coupling of alpha-bungarotoxin (αBgt) to immunoglobulin. The crude venom of *Bungarus multicinctus* was obtained from Miami Serpentarium Laboratories (Lot BM 31–1) and purified on CM-Sephadex C-50 (Pharmacia) according to the procedure described by Lee *et al.* (1972). Further purification of αBgt was carried out by chromatography on Sephadex G-50 M (Pharmacia) and carboxymethylcellulose CM-32 (Whatman). αBgt was iodinated to specific activities of either 10–30 Ci/mmol by the ICI method (Vogel, Sytkowski & Nirenberg, 1972) or 60–80 Ci/mmol by the chloramine-T method (Hunter & Greenwood, 1962). Free iodine was removed by chromatography on Biogel P-4 (Biorad). Rabbit anti-southern bean mosaic virus IgG immunoglobulin (anti-SBMV IgG) was obtained from immunized rabbits (Matter *et al.*, 1972) by ammonium sulphate precipitation of the serum followed by chromatography on DEAE–Sephadex A-50 (Pharmacia) according to Harboe & Ingild (1973).

Two kinds of complexes were made with IgG and αBgt: (a) that used to inhibit the subsequent binding of ^{125}I-labelled αBgt to the nerve terminals and consisting of IgG, αBgt and traces of ^{125}I-labelled αBgt; (b) that used for direct labelling of the nerve terminals and consisting of IgG and ^{125}I-labelled αBgt. (a) 0·1 ml of 1·15 mM toluylen-2,4-diisocyanate (TDIC, Merck) in dioxane was added to 1 ml of 0·0 5M Na-phosphate buffer (pH 7) containing anti-SBMV IgG (130 nmol), αBgt (130 nmol) and ^{125}I-labelled αBgt (0·05–2·0 nmol). After incubation at room temperature (60 min), the mixture was dialysed (60 min) against 0·05 M Na-phosphate buffer (pH 7) to remove free TDIC. The small precipitate formed upon addition of TDIC was removed by centrifugation at 1600 *g* for 5 min and the supernatant chromatographed (Fig. 1) on Sepharose 6B (Pharmacia). The fractions containing the complexes were pooled and concentrated on Diaflo membranes (UM-10; Amicon) using a micro-ultrafiltration system (Model 8MC, Amicon). Depending upon the batch of ^{125}I-labelled αBgt used, specific activities of 0·01–0·3 μCi/OD280 were obtained. A radioimmunoelectrophoresis of the concentrated material was performed as described in the legend of Fig. 2. In addition, the

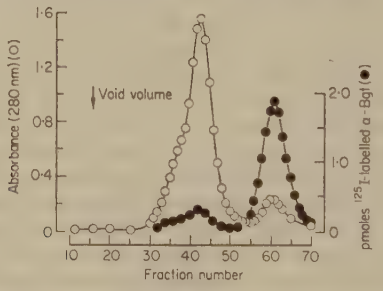

FIG. 1. Gel filtration of a complex made of IgG, αBgt and traces of ^{125}I-labelled αBgt on Sepharose 6B. The complex prepared as described in the Materials and Methods section was chromatographed at room temperature on a column (1·2 × 50 cm) equilibrated in 0·05 M Na-phosphate buffer (pH 7) at a flow rate of 8 ml/hr. 0·9 ml fractions were collected. The amount of ^{125}I-labelled αBgt was determined by counting 100 μl samples in a gamma counter (Model 5130 Packard). On separate runs and in identical conditions, the column was calibrated for IgG (peak tube number 42), αBgt (peak tube number 57) ^{125}I-labelled αBgt (peak tube number 60) and free ^{125}I (peak tube number 60).

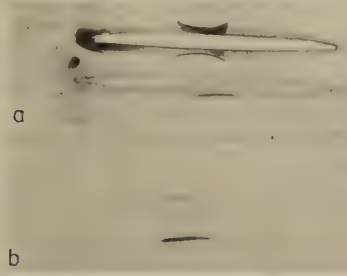

FIG. 2. Immunoelectrophoresis performed in a gel consisting of 1% agarose in 0·054 Na–barbital buffer (pH 8·6). Fifty micrograms of purified rabbit IgG were placed in the upper hole; 0·05 OD 280 αBgt–IgG complex containing 30 pmol αBgt and 0·02 pmol ^{125}I-labelled αBgt and having a specific activity of 0·02 μCi/OD 280 in the central hole and 1 nmol free ^{125}I-labelled αBgt (specific activity 2·7 mCi/mmol) in the lower hole. The electrophoresis was run for 20 min at room temperature under constant current (20 mA) and using a Multiphor Electrophoresis equipment (LKB, model 2117). Sheep anti-rabbit immunoglobulin serum was then added in the upper groove and anti-αBgt serum in the lower groove. The slide was incubated at 4°C for 24 hr. The agar gel was washed for 24 hr in PBS, dried, stained with Ponceau red (a) and exposed to X-ray film (b).

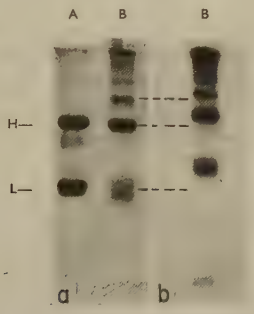

FIG. 3. (a) Electrophoresis of IgG (A) and ^{125}I-labelled αBgt–IgG complex (B) in a 12·5% acrylamide, 0·1% SDS slab gel 2 mm thick and 8 cm high. Seventeen micrograms of sheep anti-rabbit immunoglobulin IgG and 17 μg of αBgt–IgG complex (pool of fractions 31–50 after concentration, Fig. 1) were boiled 3 min in 20 μl of sample buffer containing glycerol 10% v/v, β-mercaptoethanol 5% v/v and SDS 3% w/v. Electrophoresis at 15 mA in the stacking gel, 25 mA in the running gel for 3 hr at room temperature. Coomassie blue staining. H, Heavy chains from IgG; L, light chains. (b) Radioautograph of the electrophoresis shown in (a). For details see the Results section. The traces of radioactivity detected at the bottom of the gel are probably due to free ^{125}I.

complex was analysed by polyacrylamide gel electrophoresis according to Laemmli (1970) and exposed to X-ray film (Kodirex, Kodak) (Fig. 3). (b) 130 nmol of IgG were mixed with 2–3 nmol of ^{125}I-labelled αBgt and coupled with TDIC as described in (a). Depending upon the batch of ^{125}I-labelled αBgt used, specific activities of 0·3–2·4 μCi/OD 280 were obtained.

A complex of IgM and αBgt was made in the following way: 2·6 nm of IgM were mixed with 18·7 nmol of αBgt and 2·5 nmol of ^{125}I-labelled αBgt and coupled with TDIC as described above for IgG. A specific activity of 2·5 μCi/OD 280 was obtained. The IgM used was obtained from the sera of mice bearing a plasmacytoma 104 E and purified on Sepharose 6B.

Preparation and labelling of antibodies directed against neurotoxins. (a) *Immunization.* Rabbits were immunized with cobra toxin coupled to haemocyanin in the following way: 2·5 mg α-neurotoxin purified from the venom of *Naja naja siamensis* (Lot NS2S from Miami Serpentarium Laboratories) according to Cooper & Reich (1972) were dissolved at room temperature with 2·5 mg keyhole limpet haemocyanin (KLH,

Schwarz/Mann) in 2·2 ml 0·1 M Na–phosphate buffer (pH 7). After dropwise addition of a 1% glutaraldehyde solution (10 μl/mg protein) under constant stirring at room temperature, the mixture was left for 3 hr, then dialysed against 0·15 M NaCl, 0·01 M Tris–HCl (pH 7·4) in order to eliminate the excess glutaraldehyde. A small precipitate was removed by centrifugation at 17,300 *g* for 10 min. Coupled cobra toxin was injected (1 mg toxin per rabbit) intramuscularly along the back in an emulsion with Freund's complete adjuvant. The animals were immunized with this same material four times at 2 week intervals. They were then boosted with 0·1 mg cobra toxin in phosphate-buffered saline (PBS). Rabbits were immunized with αBgt coupled to haemocyanin by following the same protocol.

(b) *Purification*. Fifteen milligrams of antitoxin IgG, obtained as described for anti-SBMV IgG, were adsorbed on a Sepharose 4B column containing covalently bound cobra toxin prepared according to Klett *et al.* (1973) and equilibrated in 0·13 M NaCl, 0·02 M Tris–HCl (pH 7·4). The resin was washed successively with the initial buffer and 2·0 M NaCl. Then the purified antitoxin IgG was eluted with 4·0 M $MgCl_2$ as suggested by Détrait & Boquet (1972), dialysed at 4°C against PBS and concentrated to 2–3 mg/ml on Diaflo membranes (UM-10, Amicon).

(c) *Iodination*. Purified antitoxin IgG was labelled with ^{125}I to specific activities of 30–70 Ci/mmol by the ICI method of McFarlane (1958) modified as follows: 27·5 nmol of ICI in 5 μl methanol were incubated at 0°C with 0·5–1·0 mCi of carrier-free Na ^{125}I (Amersham) in 75 μl of a solution 33 mM in HCl and 170 mM in NaCl. After 4 min this solution was added to 250 μl of 0·4 M NH_4Cl adjusted to pH 8·95 with NH_4OH and containing 0·75 mg of antitoxin IgG. After 2 min at 0°C, the iodination reaction was terminated by the addition of 20 μl of 0·1 M $Na_2S_2O_5$ and 20 μl of 0·1 M NaI. Free iodine was removed by gel filtration on Biogel P4 (Biorad) equilibrated in 10 mM Na-phosphate buffer, 10 mM NaCl (pH 7·4).

(d) *Characterization*. IgG antitoxin activity of immunoglobulins purified on affinity chromatography was tested by immunodiffusion. 250–500 μg of IgG were added to 3 ml of a 1% agarose solution in PBS. The resulting mixture was spread on a slide (76 × 27 mm). Holes made in the gel were filled with different dilutions of cobra toxin (0·6–10 μg/ml in PBS). The precipitation rings were read after 24 hr. The toxin binding of ^{125}I-labelled IgG was tested as follows: 16 μg of ^{125}I-labelled IgG antitoxin were mixed with 420 μg purified antitoxin IgG and an immunodiffusion test was performed as indicated above. After 24 hr, the gel was extensively washed in PBS, dried and exposed for 15 min on an X-ray film (Fig. 4). In order to check a possible unspecific trapping of ^{125}I-labelled IgG antitoxin, 14 μg of ^{125}I-labelled IgG antitoxin were added to 0·3 ml anti-IgM serum, mixed with agarose solution and spread on a slide as described above. The holes were filled with different dilutions of IgM (0·12–4 mg/ml in PBS). In these conditions, the precipitation rings were not labelled even after 7 hr of exposure to an X-ray film.

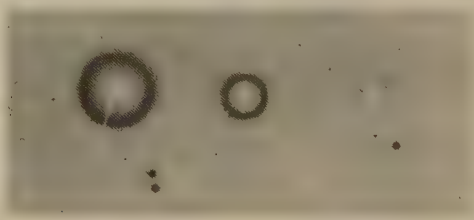

FIG. 4. Agar gel radioimmunodiffusion test of ^{125}I-labelled antitoxin (specific activity 70 Ci/mmol) with different concentrations of cobra toxin: 1:500, 1:1000, 1:2000, 1:4000, 1:8000 dilutions of a 5 mg/ml cobra toxin solution. For details see the Materials and Methods section.

RESULTS

(a) *Covalent coupling of αBgt to IgG*

Fig. 1 illustrates that in a complex consisting of IgG, αBgt and traces of ^{125}I-labelled αBgt, less than 5% of αBgt molecules used initially in the coupling procedure are covalently linked with IgG molecules and recovered with the complexes in gel filtration on Sepharose 6B (fractions 30–50). This proportion is calculated by comparing the quantity of ^{125}I-labelled αBgt present in this complex (2·5 pmol) with the total amount of ^{125}I-labelled αBgt (75 pmol) added to the reaction mixture before coupling and assuming that iodinated and noniodinated αBgt are coupled with IgG to the same extent. As judged by the profile on gel filtration, the

complex is heterogeneous in size. It is at least as large as IgG molecules (see calibration with IgG in Fig. 1).*

This complex and rabbit IgG were compared on immunoelectrophoresis (Fig. 2a). Both show a precipitation line with sheep anti-rabbit immunoglobulin serum. The same complex and ^{125}I-labelled αBgt were also compared on this immunoelectrophoresis. Both show a precipitation line with rabbit anti-αBgt serum. These results indicate that antigenic determinants of both IgG and αBgt molecules are present in the complex. Moreover, it can be concluded that ^{125}I-labelled αBgt is covalently linked to IgG since the lines resulting from the complex are revealed on the radioautograph and the lines due to free and bound ^{125}I-labelled αBgt do not migrate to the same position (Fig. 2b).

This complex and sheep IgG were compared using sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis in reducing conditions (Fig. 3). The major bands of the complex migrate the same distance as the heavy (H) and light (L) chains of IgG. As only 5% of the IgG molecules are coupled with αBgt (mol. wt 8000), one cannot expect to find visible protein bands corresponding to molecular weights higher than H (mol. wt 55,000) and L (mol. wt 22,000) chains. However, as the complexes are radioactively labelled, bands corresponding to molecular weights higher than H (mol. wt $\geqslant$63,000) and L (mol. wt $\geqslant$30,000) chains should be revealed on a radioautograph. This is clearly shown on Fig. 3b.

(b) *In vitro inhibition of ^{125}I-labelled αBgt binding on mouse diaphragms*

These experiments were performed with a complex consisting of IgG, αBgt and traces of ^{125}I-labelled αBgt. A diaphragm was incubated with this complex to determine if this treatment prevents subsequent labelling with ^{125}I-labelled αBgt. In one experiment a diaphragm incubated with ^{125}I-labelled αBgt (50 pmol, specific activity 30 Ci/mmol) exhibits a strong labelling of the nerve terminal region after exposure on X-ray films. A preincubation with high concentrations of unlabelled αBgt (12 nmol) prevents the labelling of this diaphragm. The same inhibition is also obtained if the diaphragm is incubated first with an IgG–αBgt complex containing 12 nmoles αBgt whereas IgG alone has no effect on a subsequent labelling with ^{125}I-labelled αBgt. One has to exclude the possibility that even a small fraction of the 12 nmoles αBgt present in the complex is liberated during incubation and responsible for the inhibitory effect described above. For this purpose, an ^{125}I-labelled αBgt–IgG complex (17·2 OD 280 containing 180 pmol ^{125}I-labelled αBgt), purified on a Sepharose 6B column and incubated for 2 hr at 37°C in 2 ml Eagle's medium containing 10% FCS was tested. The possible release of minimal amounts of ^{125}I-labelled αBgt (0·1 pmol) was tested in the following way: the incubated complex was chromatographed a second time on the Sepharose 6B column. Six per cent of the total counts applied to the column were eluted in fractions 55–65 which correspond to the positions where ^{125}I-labelled αBgt or free ^{125}I are eluted (see legend of Fig. 1). The material present in these fractions was incubated with a preparation of purified AChR.† It did not show any binding to AChR. Thus the radioactive material contained in these fractions is considered not to be ^{125}I-labelled αBgt but free ^{125}I. The *in vitro* inhibitory effect of α-Bgt-IgG complex on subsequent labelling with ^{125}I-labelled αBgt is therefore not due to a contamination of the complex with free αBgt.

(c) *In vivo labelling of acetylcholine receptors (AChR) on mouse diaphragm*

These experiments were performed in order to see if direct labelling of AChR were possible after an i.v. injection of complexes made of IgG and ^{125}I-labelled αBgt. This is illustrated in

* The molecular weight of αBgt is only 8000. Thus a complex made of toxin and IgG is not expected to migrate on Sepharose 6B differently from IgG.

† AChR was purified from *Torpedo marmorata* according to the method described by Klett *et al.* (1973) for *Electrophorus electricus*. AChR activity was tested with the DEAE-filter disc assay described by the same authors. 0·1 pmol AChR of known activity was used in this test.

Fig. 5 in which the labelling obtained with these complexes is compared (b) with the one obtained after *in vivo* injection of ^{125}I-labelled αBgt (a). In both cases the animals did not show any sign of paralysis after 3 hr and were killed. On the diaphragms, the regions of nerve terminals appear labelled. These results indicate that not only small molecules (αBgt mol. wt 8000) but also molecules as large as IgG (mol. wt 150,000) can enter the synaptic cleft. One experiment was performed in order to see if molecules even the size of IgM (mol. wt 900,000) were able to enter the synaptic cleft. The complex made as described in the Materials and Methods section was used in an experiment illustrated in Fig. 5c. Although the animal was killed 2½ hr after the injection, it appears that the complex, at that time, has already reached the nerve terminal regions. Contrary to the experiments described under (b), the liberation of small amounts of ^{125}I-labelled αBgt cannot be excluded in these *in vivo* cases.

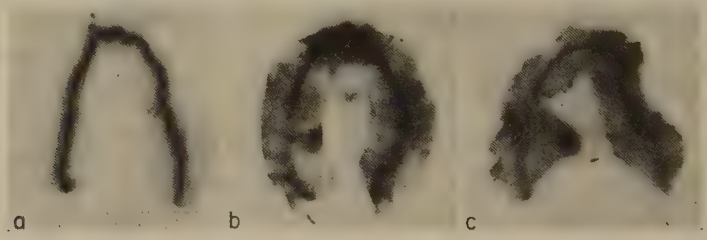

Fig. 5. (a) X-ray film exposed 4 days to the diaphragm of a 20 g Swiss mouse injected i.v. with 1·25 nmol ^{125}I-labelled αBgt (specific activity 30 Ci/mmol). The animal was killed 2½ hr after the injection. The diaphragm was immediately dissected out, extensively washed in PBS, dried on filter paper and placed on an X-ray film. (b) X-ray film exposed 2 days to the diaphragm of a 20 g C3H/C57 mouse injected i.v. with 6·3 OD 280 ^{125}I-labelled αBgt–IgG complex (specific activity 2 μCi/OD). The animal was killed 3½ hr after the injection and the diaphragm was handled as described in (a). (c) X-ray film exposed 5 days to the diaphragm of a 19 g Swiss mouse injected i.v. with 2·1 OD 280 ^{125}I-labelled αBgt–IgM complex (specific activity 2·5 μCi/OD). The animal was killed 2½ hr after the injection and the diaphragm handled as described in (a).

(d) *Detection by ^{125}I-labelled anti-cobra toxin of cobra toxin present on mouse diaphragms*

Another way of checking whether molecules the size of IgG can penetrate into the synaptic cleft is to observe if radioactively labelled anti-cobra toxin IgG binds to cobra toxin molecules themselves linked to AChR *in situ*. Fig. 6a illustrates this experiment. A mouse which had first received a sublethal dose of cobra toxin was injected 2 hr later with ^{125}I-labelled antitoxin. Three hours after this second injection, the animal was killed and the diaphragm exposed on X-ray film. Labelling of the nerve terminal region is obvious on this film. In a control experiment (Fig. 6b), a mouse having not received any cobra toxin was injected with a similar dose of ^{125}I-labelled anticobra toxin. In this case, the nerve terminal region of the diaphragm is not labelled. These results demonstrate again that, *in vivo*, molecules the size of IgG can reach to synaptic cleft.

The following experiment was designed in order to check if this also occurs *in vitro*. A mouse received a lethal dose of cobra toxin. The diaphragm was taken out after death of the animal and incubated in a medium containing ^{125}I-labelled anticobra toxin as described in the Materials and Methods section. After exposure on X-ray film, the nerve terminal region of the diaphragm appears labelled (Fig. 6c) whereas a normal diaphragm incubated in the same conditions with ^{125}I-labelled anticobra toxin does not exhibit any labelling (Fig. 6d).

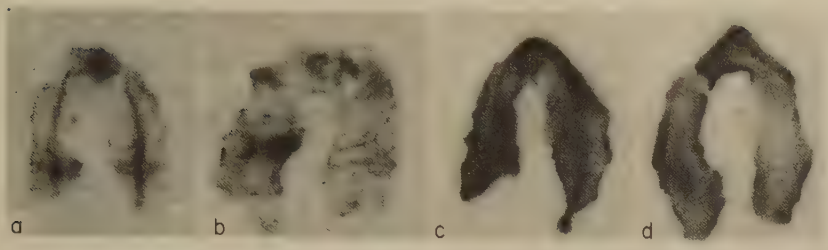

FIG. 6. (a) X-ray film exposed for 3 days to the diaphragm of a 16 g Swiss mouse injected i.v. with a sublethal dose of cobra toxin (0·08 μg/g) and, 2 hr later, with 800 pmol of ^{125}I-labelled antitoxin (specific activity 26 Ci/mmol). The animal was killed 3 hr after the second injection. The diaphragm was immediately dissected out, extensively washed in PBS, dried on filter paper and placed on X-ray film. (b) X-ray film exposed for 3 days to the diaphragm of a 25 g Swiss mouse injected i.v. with 480 pmol of ^{125}I-labelled antitoxin (specific activity 26 Ci/mmol). The animal was killed 3 hr after the injection and the diaphragm handled as described in (a). (c) X-ray film exposed for 18 hr to the diaphragm of an 18 g Swiss mouse injected i.v. with a lethal dose of cobra toxin (0·1 μg/g). This diaphragm was dissected out immediately after death (30 min after the injection), washed extensively for 1 hr in PBS and then incubated for 1 hr at 37°C in 2 ml Eagle's medium containing 10% FCS and 100 pmol ^{125}I-labelled antitoxin (specific activity 30 Ci/mmol). After extensive washing in PBS (1–3 hr in 500–1000 ml), the diaphragms were dried on filter paper and placed on X-ray film. (d) X-ray film exposed for 18 hr to the diaphragm of a normal 20 g Swiss mouse. This diaphragm was dissected out immediately after the animal was killed, washed extensively for 1 hr in PBS and then incubated with 100 pmol ^{125}I-labelled antitoxin as described in (c).

Thus, molecules the size of IgG are able to diffuse into the synaptic cleft during *in vitro* incubation of a diaphragm.

DISCUSSION

Recent evidences have shown that antibodies to AChR are present in the serum of almost all myasthenic patients. On human tissue sections these antibodies block the binding of αBgt to AChR (Bender *et al.*, 1975). *In vitro* they bind to solubilized human receptors (Almon, Andrew & Appel, 1974; Almon & Appel, 1975; Lindstrom, in press). Nevertheless one does not know yet if these circulating antibodies are capable, *in vivo* and *in situ*, of directly blocking the receptor. First of all one can wonder whether immunoglobulins are able or not to penetrate the intact neuromuscular junction. The experiments described in this paper were designed to answer that question.

Mouse diaphragms were chosen because of the regular arrangement of their nerve terminals. Waser & Lüthi (1956) were the first to show, on mouse diaphragms, that nerve terminals labelled with radioactive neuromuscular blocking agents are easily seen. This organ can therefore be used to analyse morphologically a phenomenon occurring at the molecular level.

Complexes made of αBgt covalently coupled to IgG or IgM were used to test the accessibility to immunoglobulins of AChR located in the neuromuscular junction. The data presented here show that the coupling procedure used is satisfactory and that the radioactivity of the complexes obtained is really due to the iodinated αBgt. Moreover, these complexes are still able to bind AChR as judged by the experiments in which they prevent subsequent labelling of the nerve terminals with iodinated αBgt. Finally, these complexes are shown to be stable in the *in vitro* conditions. In the *in vivo* conditions, however, a possible release of small amounts of αBgt through enzymatic hydrolysis cannot be entirely excluded. In this context the demonstration reported here that ^{125}I-labelled anticobra toxin antibodies can

penetrate the neuromuscular junction represents a better evidence that molecules the size of immunoglobulins can enter *in vivo* the synaptic cleft.

These results oblige us to assume that either the complexes made of αBgt and IgG or the anticobra toxin antibodies leave the vascular compartment, diffuse into the extracellular space and reach AChR located at the top of the folds of the postsynaptic membrane and that this process is rapid. Actually our data show that the diaphragms are labelled in less than 3 hr after the injection. Of course a sudden and large increase in blood vessel permeability occurring when the animal is sacrificed could explain such a rapid diffusion. We have tried to minimize the role of such a possible factor by washing extensively the diaphragm less than 3 min after the death of the mouse.

The present results favour the possibility that molecules the size of antibodies can reach AChR *in situ*. We are trying now to check whether anti-AChR antibodies found in the sera of myasthenic patients or in the sera of animals with experimental MG can be detected within the neuromuscular junction (work in preparation). It should be, however, well noticed that even if the presence of anti-AChR antibodies can be demonstrated within the nerve terminals, this will not imply that they are responsible for the signs of paralysis observed.

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Study of two different subpopulations of anti-acetylcholine receptor antibodies in a rabbit with experimental autoimmune myasthenia gravis*

Two different subpopulations of anti-acetylcholine receptor antibodies were studied during the evolution of experimental autoimmune myasthenia in one rabbit immunized with *Torpedo* acetylcholine receptor. The results show that the subpopulation of antibodies directed against the toxin-binding site of the receptor might play a role in the appearance of the paralysis observed in this particular case.

1. Introduction

Numerous animal models sharing many similarities with human myasthenia gravis have been reported. Rabbits [1–4], rats [2, 5], guinea pigs [2, 5], monkeys [6], goats [2] and mice [7, 8] were immunized with acetylcholine receptor (AcChR) purified from either fish electric organs (*Electrophorus electricus* [2–5], *Torpedo californica* [5, 6, 8], *Torpedo marmorata* [1]) or rat-denervated muscles [7]. These animals showed muscle weakness due to impaired neuromuscular transmission. Their sera contained high concentrations of anti-AcChR antibodies. These antibodies are usually measured with a radioimmunoassay which detects only antibodies directed against other sites than the acetylcholine-binding site of the receptor. The pathogeny of the paralysis is still debated. According to one hypothesis [9], antibodies could act as acetylcholine competitive blocking substances and thereby impair the neuromuscular transmission. One should state more precisely that, theoretically, only antibodies directed against the acetylcholine-binding site of the receptor could play this role. The recent demonstration by Lindstrom of the presence of these anti-AcChR antibodies, although in small quantities, supports this hypothesis, but this author did not attempt to correlate their levels with the clinical picture [2].

In the present report, we have followed these anti-AcChR antibodies during the evolution of experimental myasthenia in a rabbit immunized with AcChR purified from *Torpedo marmorata*. These antibodies were detected by measuring their ability to inhibit ^{125}I-labeled α-toxin binding on solubilized AcChR. The results show that, in this case, the amount of antibodies directed against the toxin-binding site increases strongly just before the appearance of paralysis whereas the level of antibodies directed against other sites is not significantly higher at that time. In addition, anti-*Torpedo* AcChR antibodies inhibit ^{125}I-labeled α-toxin binding also on mammalian muscle AcChR *in situ* (mouse diaphragm). This latter preparation reflects better than solubilized AcChR, what might happen *in vivo*.

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Abbreviations: AcChR: Acetylcholine receptor PBS: Phosphate-buffered saline α-Bgt: Alpha-Bungarotoxin

2. Materials and methods

2.1. Toxins

The main neurotoxin (α-toxin) from the venom of *Naja naja siamensis* (lot NS2S from Miami Serpentarium Laboratories) was isolated according to the method of Cooper and Reich [10]. α-Bungarotoxin (α-Bgt) was purified from the venom of *Bungarus multicinctus* (lot BM 31-1 from Miami Serpentarium Laboratories) as described by Lee et al. [11] with the modifi-

cations reported by Monnier and Fulpius [12]. Both toxins were radioiodinated to specific activities of 10–50 Ci/mmol using the method described by Devreotes and Fambrough [13].

2.2. AcChR

Purified AcChR was obtained from frozen electric organs of *Torpedo marmorata* using the method described by Klett et al. [14] for *Electrophorus electricus* with the two following modifications: (a) Triton X-100, at a concentration of 1 %, (v/v), was used as the solubilizing agent, (b) the DEAE-cellulose chromatography step was omitted. With this procedure, specific activities of 2.5–5.0 nmol ^{125}I-labeled α-Bgt bound per mg protein were obtained, assuming that 1 mol AcChR binds 1 mol of toxin.

2.3. Immunization of the rabbit

The rabbit was immunized according to the following procedure: the first injection (day 1) was made in complete Freund's adjuvant, the second one (day 10) in incomplete Freund's adjuvant, the two subsequent ones (days 18 and 29) in phosphate-buffered saline (PBS). The rabbit was further immunized in complete and incomplete Freund's adjuvant (days 50 and 57) and bled to death at day 66.

2.4. Detection of anti-AcChR antibodies

2.4.1. Anti-AcChR antibodies directed against sites other than the toxin-binding site

These were detected with a radioimmunoassay similar to the one developed by other authors [15]. In this radioimmunoassay, a complex of purified AcChR and ^{125}I-labeled α-Bgt was used as antigen. This complex, 8 x 10^{-9} M in buffer A (0.05 M, Tris-HCl, pH 7.4, 0.1 M NaCl, 1 % Triton X-100 (v/v)), was obtained by incubating purified AcChR with a 5-fold excess of ^{125}I-labeled α-Bgt and removing excess α-Bgt by Sephadex G-100 chromatography. The complex (0.08 pmol) was diluted in 0.2 ml of buffer A. Control serum (2 μl) from a rabbit immunized with ferritin and increasing amounts of each rabbit anti-AcChR serum (5 to 20 μl) were added to this mixture and incubated for 1 h at 37 °C. Depending on the titers of anti-AcChR antibodies present in these latter sera, they were first diluted 1:10, 1:100, and 1:1000 with PBS. At the end of the incubation period, the amount of sheep anti-rabbit Ig serum required to precipitate the rabbit Ig was added. The resulting mixture was incubated for one more hour at 37 °C. The tubes were then placed for 2 h at 0 °C. The precipitates were centrifuged (1700 x g, 5 min, 4 °C) and washed three times with ice-cold PBS. The radioactivity of the pellet was measured in a gamma counter (Autogamma Scintillation Spectrometer Packard 5160). The antibody titers were expressed in nmol ^{125}I-labeled α-Bgt-AcChR complex precipitated per ml serum.

2.4.2. Anti-AcChR antibodies directed against the toxin-binding site

These were detected by measuring the inhibition of ^{125}I-labeled α-toxin fixation on the purified AcChR. Rabbit anti-AcChR serum (0, 5, 10, 20 μl diluted 1:100 in PBS and 5, 10 μl diluted 1:10) was added to six tubes, each containing 0.2 ml buffer B (0.05 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.1 % Triton X-100 (v/v)), 0.1 ml rabbit immune serum, and 0.01 ml ^{125}I-labeled α-toxin (2.0 pmol). The resulting mixture was thoroughly mixed. Then, AcChR (1.5 pmol in 0.01 ml buffer A) was added to each tube. The six tubes were incubated at room temperature for a period of 15–20 h. Thereafter, the six samples were chromatographed simultaneously on Ultrogel AcA 44 (LKB, Bromma, Sweden) at room temperature. For this purpose we used six identical plastic columns (0.8 cm x 28.0 cm) equilibrated with buffer B. The chromatographies were performed at a rate of 12 ml/h under constant hydrostatic pressure. Fractions of 0.6 ml were collected manually, and the radioactivity measured as previously described. Under these conditions, the radioactive material loaded on each column was recovered in two distinct peaks corresponding to bound and free toxin. The amount of ^{125}I-labeled α-toxin not specifically trapped in the first peak, was measured in a separate run performed in the absence of AcChR. Under these conditions, this amount corresponded to 2 % of the counts loaded. Accordingly, in each run, 2 % were subtracted from the counts measured in the first peak. The relative amount of antibodies directed against the toxin-binding site in a given amount of serum was expressed as percentage inhibition of toxin binding. This percentage was derived in the following way: the amount of ^{125}I-labeled α-toxin bound to AcChR in presence of serum (X) was subtracted from the amount of ^{125}I-labeled α-toxin bound to AcChR in its absence (Y). This quantity (Y-X) was then divided by Y to give the percentage of inhibition of toxin binding. With increasing amounts of serum, curves as those shown in Fig. 1 were obtained. The percentage of toxin inhibition per 0.1 μl of each serum was derived from the initial slope.

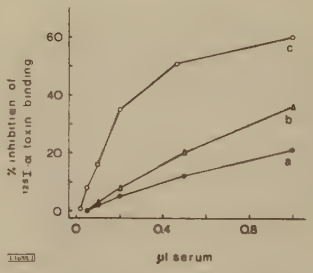

Figure 1. Inhibition curves of ^{125}I-labeled α-toxin binding to purified AcChR by anti-AcChR sera taken at three different times of the immunization procedure. Toxin, receptor and serum were incubated as described in Sect. 2.4.2. The curves a, b and c correspond to sera obtained at days 24, 37 and 66, respectively (see Fig. 2).

2.5. Mouse diaphragms

Diaphragms from two-month-old C3H mice were dissected and washed in PBS at room temperature. A portion of about 0.7 cm was cut on each side of the diaphragm. Each fragment was incubated in a plastic tube containing 1 ml anti-*Torpedo* AcChR serum or control serum. Complement was previously inactivated by heating the serum at 56 °C for 30 min. Dilutions (1:2–1:20) of the anti-AcChR serum were made with control serum. After 1 h incubation at 37 °C, ^{125}I-labeled α-toxin (7 pmol in 10 μl PBS) was added to each tube and

the incubation was continued for 30 min. The portions of diaphragm were rinsed with PBS, washed twice for 30 min at room temperature in 250 ml PBS, dried on filter paper and cut into 10 to 12 strips, 0.5 mm in width. The sections were made perpendicular to the orientation of the muscle fibers as described by Green et al. [16]. The region of the nerve terminals was located between strips 5 and 9. The radioactivity present in each strip was measured in an Autogamma Scintillation Spectrometer (Packard 5160).

3. Results

When inhibition of toxin binding was measured with increasing amounts of anti-AcChR serum, curves like those shown in Fig. 1 were obtained. The initial slopes of these curves were used to calculate toxin-binding inhibition. Values of 3.0, 5.3 and 25 % per 0.1 µl serum were derived for the antisera taken at days 24, 37 and 66 of the immunization procedure (curves a, b and c in Fig. 1).

Anti-AcChR antibody levels measured by radioimmunoassay during the immunization procedure are shown in Fig. 2a. At day 66, when paralysis was fully developed, the level was 7 nmol/ml. This value is 1.4 times higher than the maximal level measured previously (5.1 nmol/ml at day 24). Inhibition of toxin binding at different times of the immunization procedure is shown in Fig. 2b. At day 64, a level of 37 % per 0.1 µl serum was reached. This value is 7 times higher than the highest one measured previously (5.3 %/0.1 µl serum at day 37). At day 66, the inhibition was 25 %.

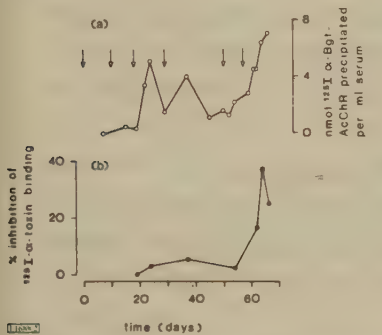

Figure 2. Anti-AcChR antibodies in the serum of a rabbit at different times of the immunization procedure. Days of AcChR injection are indicated by arrows. Injections were performed as described in Sect. 2.3. The animal was bled to death at day 66. (a) Titers of antibodies directed against other sites than the toxin-binding site, expressed in nmol ^{125}I-labeled α-Bgt-AcChR complex precipitated/ml serum. (b) Inhibition of ^{125}I-labeled α-toxin binding by antibodies directed against the toxin-binding site, expressed in percentage/0.1 µl serum.

When portions of mouse diaphragms were incubated successively with control serum and ^{125}I-labeled α-toxin, the region of the nerve terminals was radioactively labeled as shown in Fig. 3, curve c. With the anti-AcChR serum taken at day 66 (anti-serum 66) only background radioactivity was detected in this region (curve a). With anti-serum 66 diluted 1:10 with control serum, a discrete labeling appeared (curve b). Other dilutions of anti-serum 66 were also tested. The respective quantities of ^{125}I-labeled α-toxin bound to the nerve terminal regions are given in Table 1. With a 1:20 dilution, the quantity of ^{125}I-labeled α-toxin bound to the nerve terminal region is comparable to the one measured with control serum. The amount of toxin bound in presence of anti-AcChR serum taken at day 24 (anti-serum 24) is also shown in Table 1.

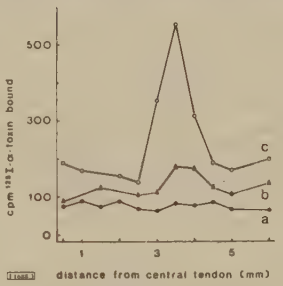

Figure 3. ^{125}I-labeled α-toxin (cpm) bound to muscle strips obtained by cutting a portion of mouse diaphragm from the central tendon to rib insertion. The three profiles correspond to portions of diaphragms incubated with (a) anti-AcChR serum 66, (b) anti-AcChR serum 66 diluted 1:10 and (c) control serum.

Table 1. ^{125}I-labeled α-toxin (cpm) bound to the nerve terminal region of mouse diaphragm pre-incubated with control serum or anti-AcChR sera

Serum	Dilutions	^{125}I Radioactivity (cpm)
Control	–	1551
Anti-AcChR 66	–	387
	1:2	421
	1:4	448
	1:8	518
	1:10	697
	1:20	1314
Anti-AcChR 24	–	881

4. Discussion

As also reported by others [17, 18], rabbits immunized with *Torpedo* AcChR developed a flaccid paralysis and rising levels of anti-AcChR antibodies measured by radioimmunoassay. The first signs of paralysis usually appeared about one week after the second or third injection. In the case reported here, however, there were no signs of paralysis at that time, though high levels of antibodies were present. As paralysis did not develop even after two boosts in PBS, we re-injected AcChR in Freund's adjuvant. One week later the rabbit was paralyzed.

Two populations of anti-AcChR antibodies were measured during the immunization procedure. The level of antibodies

directed against sites other than the toxin-binding site (measured by radioimmunoassay), was only 1.4 times higher at the onset of paralysis than three weeks before, whereas, at that time, anti-AcChR antibodies directed against the toxin-binding site caused a 7 times higher toxin-binding inhibition.* This latter subpopulation of anti-AcChR antibodies is of special interest since these antibodies could well act as curare-like agents responsible for the neuromuscular blockade observed [9]. There are no reports published on evaluation of this antibody subpopulation during immunization of an animal with AcChR. The only data reported are inhibitions produced by sera obtained at one single time [2, 19, 20]. Our assay rests on the ability of these antibodies to inhibit α-toxin binding to soluble AcChR, conditions similar to those used by Almon and Appel to detect anti-AcChR antibodies in human serum [21]. The inhibitions observed during the evolution of the disease in the rabbit described here, seem to indicate that there is a correlation between the levels of anti-AcChR antibodies directed against the toxin-binding site and the appearance of signs of paralysis after reimmunization. The inhibition at day 66 (25 %) was inferior to the one measured two days before (37 %). This decrease could be explained by the binding of anti-AcChR antibodies to AcChR in the neuromuscular junction.

The antiserum characterized by a 25 % inhibition of toxin binding on solubilized AcChR prevents toxin binding also on mouse AcChR located within the intact muscle membrane. This is another evidence compatible with the hypothesis that anti-AcChR antibodies are able to act as curare-like agents and might cause the neuromuscular block observed in experimental autoimmune myasthenia. This latter finding also shows that anti-*Torpedo* AcChR serum cross-reacts with mammalian muscle AcChR, at least at the toxin-binding site. This should be the most constant part of the receptor molecule among different animals. The diaphragm preparation represents a way of testing what might happen *in vivo* better than the solubilized receptor since, with the receptor *in situ*, it is less probable that anti-AcChR serum would inhibit toxin binding at other than the toxin-binding site. In intact skeletal muscles, a major part of the receptor is anchored within the membrane, and the hydrophilic part of the molecule only, including the toxin-binding site, is accessible to circulating antibodies.

According to the present data, the diaphragm preparation is not sensitive enough to be used as an assay to test anti-AcChR antibodies directed against the toxin-binding site, as it was restricted to sera with inhibitions superior to 3 %/0.1 μl serum. The inhibitions measured on mouse diaphragms with dilutions of antiserum 66 show that this serum is about 10 times more effective in inhibiting toxin binding than antiserum 24. This difference is comparable to the respective inhibitions measured on soluble *Torpedo* AcChR (25 % and 3 % for antisera 66 and 24). Thus, these experiments would indicate that in both cases, the effect of the same subpopulation of anti-AcChR antibodies was measured.

* This inhibition, measured with anti-AcChR serum, is due to antibodies directed against the toxin-binding site since we observed the same effect with purified IgG.

In conclusion, the present observation suggests that one of the two populations of anti-AcChR antibodies studied, the one which inhibits toxin binding, might play a role in the appearance of the paralysis observed in this particular case.

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Note added in proof: The inhibition of toxin fixation on mouse diaphragm is due to the IgG fraction of antiserum 66 since this serum was not active after adsorption on protein A-Sepharose. Inhibition of toxin fixation was also observed when a diaphragm was incubated at 4 °C. In this case it is not probable that the AcChR-antibody complex was internalized. The inhibition observed might therefore well be due to the curare-like action of the anti-AcChR antibodies.

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DISCUSSION

In this work we have tried to analyse the possible role of anti-AcChR antibodies in the neuromuscular blockade observed in experimental autoimmune myasthenia gravis. The properties of anti-AcChR sera from rabbits immunized with solubilized and purified *Torpedo* AcChR were studied with three different AcChR preparations:

1) detergent-solubilized, purified AcChR in which antigenic sites usually buried within the membrane should be accessible to the antibodies, provided they are not masked by the detergent molecules.

2) AcChR-rich membrane fragments in which only those parts of the AcChR which are not buried within the membrane are accessible to the antibodies.

3) whole organs rich in AcChR in which only those parts of the AcChR which are on the external surface are accessible to the antibodies.

1) Anti-AcChR antibodies were first quantitated with solubilized AcChR. The amount of anti-AcChR antibodies present in a given serum or purified IgG fraction was determined by a radioimmunoassay in which the antigen is a complex made of purified AcChR and ^{125}I labeled α-Bgt. Values from 0.8 to 7 nmoles complex precipitated per ml serum were obtained in 7 rabbits at the time of appearance of signs of paralysis (Table I). According to this and as also shown in Fig. 2 of paper (2), there is no correlation between the amount of anti-AcChR antibodies present in the serum during the immunization of a rabbit and the appearance of signs of paralysis. This argues against a direct role of the anti-AcChR

antibodies in the appearance of the neuromuscular blockade. As the radioimmunoassay used does not detect anti-AcChR antibodies directed against the toxin binding site, this site being blocked by α-Bgt, it was necessary to develop a different method for measuring this subpopulation of anti-AcChR antibodies. With this second method, we measured the percentage of inhibition of ^{125}I-α-T binding to solubilized AcChR which is produced by anti-AcChR antibodies*. We showed (Fig. 1) that there are anti-AcChR antibodies which inhibit ^{125}I-α-T binding to AcChR in the sera of rabbits immunized with AcChR. According to Fig. 2 (double reciprocal plot with the same intersection on the ordinate for all curves) this inhibition is competitive. However, the inhibition does not occur exclusively at the toxin binding site since, above a certain concentration of antibodies (Fig. 1, paper {2}) the percentage of inhibition did not increase linearly and total inhibition could not be reached even at high antibody concentrations (see results).

On the other hand, our experiments with a serum raised against denatured AcChR (boiled for 10 minutes) favours the fact that the inhibition of toxin binding observed on solubilized AcChR was due to a subpopulation of anti-AcChR antibodies directed against parts of the AcChR involving the toxin binding site. As a matter of fact, this denatured AcChR no longer binds toxin. It does not induce paralysis when injected into a rabbit. The serum obtained with this denatured AcChR did not inhibit toxin binding, though it reacted to some extent with the native AcChR as measured by RIA (Table I). Thus, it might well be that in the absence of an intact toxin binding site on the injected AcChR, the rabbit does not make those antibodies which inhibit toxin binding. This is indirect evidence that the serum of a rabbit immunized with

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* α-T was used because its off-rate from the AcChR is faster than that of α-Bgt (a few hours instead of several days).

native AcChR contains a subpopulation of antibodies directed against parts of the toxin binding site.

2) We analysed the effect of anti-AcChR antibodies on toxin binding to the AcChR in its original membrane environment, that is on AcChR-rich membrane fragments. In this case, only antigenic sites not hidden within the membrane are accessible to the antibodies. We observed that a given serum is 11 times less efficient in inhibiting toxin binding to AcChR-rich membrane fragments than to solubilized AcChR. This means that antibodies against antigenic sites of the AcChR usually buried within the membrane can influence toxin binding on the solubilized AcChR. They could, for instance,induce a conformational change which could lead to a decrease in the affinity for the toxin. Thus, according to these results and as also noticed above, the inhibition of toxin binding measured on solubilized AcChR is not due exclusively to the subpopulation of anti-AcChR antibodies directed against the toxin binding site.

3) We also analysed the effect of anti-AcChR antibodies on toxin binding to AcChR located within the postsynaptic membrane of the neuromuscular junction. For that purpose we used either isolated mouse diaphragm (a) or rat nerve-diaphragm preparations (b).

a) In paper (1) we showed that molecules the size of immunoglobulins can enter the narrow synaptic cleft and reach postsynaptic membrane molecules. Results from incubations of mouse diaphragms with anti-AcChR antibodies and ^{125}I-α-T are discussed in paper (2). In particular, we showed that the antiserum taken at the time of appearance of paralysis in rabbit no 5 inhibits ^{125}I-α-T binding to the neuromuscular junction region

of mouse diaphragm.

b) Experiments performed with rat nerve-diaphragm preparations are described under methods and results. Incubations for as long as two hours with different concentrations of sera raised either against purified *Torpedo* AcChR or *Torpedo* AcChR-rich membrane fragments did not lead to a decrease of the amplitude of contraction of the diaphragm muscle.

Whereas results described under a) confirm the inhibitory effect of anti-AcChR antibodies on toxin fixation, those described under b) argue against an effect of these antibodies on neuromuscular transmission. This lack of effect can be explained in several ways:

i) the incubation time was not long enough because of the slow diffusion of the antibodies into the narrow synaptic cleft. This argument does not seem to be valid since we know that anti-AcChR antibodies completely inhibit ^{125}I-α-T binding to mouse diaphragm after a one hour incubation (see paper (2)).

ii) anti-*Torpedo* AcChR antibodies do not cross-react sufficiently with rat AcChR, although they do recognize mouse AcChR (paper (2)).

iii) anti-AcChR antibodies block only a small proportion of AcChRs and no effect on the contraction can be observed. It has indeed been shown by Albuquerque et al. (1973a) that the amplitude of the EPP measured in a rat nerve-muscle preparation starts to decrease only when more than 20% of the AcChRs are blocked with α-Bgt. In addition, on a mouse nerve-diaphragm preparation, the amplitude of contraction starts to decrease only

when more than 40% of the AcChRs are blocked with α-Bgt (Barnard et al., 1971). Thus, the method using the inhibition of muscle contraction to detect a blocking effect of the anti-AcChR antibodies on neuromuscular transmission might not be sensitive enough.

iv) anti-AcChR sera known to contain antibodies against the toxin binding site of the AcChR do not necessarily contain antibodies against the acetylcholine binding site (see below: hexamethonium and carbamylcholine do not inhibit ^{125}I-anti-AcChR binding to membrane fragments).

Points iii) and iv) will be further discussed in the conclusion in connection with results from the literature.

To analyse this problem further, we tried a different approach: instead of inhibiting binding of a small molecule (^{125}I-α-T) to the AcChR with large molecules (anti-AcChR antibodies), we tried to inhibit binding of the large molecules with small ones. For this purpose, we labeled anti-AcChR antibodies with ^{125}I and tried to inhibit their binding to membrane fragments with α-Bgt, hexamethonium or carbamylcholine. Such experiments should also tell us whether there exist, within the anti-AcChR antibody population, specific subpopulations which recognize the binding site for α-Bgt, hexamethonium or carbamylcholine. In addition, they should better reflect the direct interactions of anti-AcChR antibodies with the toxin binding site itself rather than with additional antigenic sites close to it.

The results show that α-Bgt inhibits ^{125}I-anti-AcChR binding to membrane fragments by 10-15% (Fig. 4). In view of the fact that at least 30% of the binding of the ^{125}I-anti-AcChR to the

fragments is nonspecific (Fig. 5), this percentage reflects in fact a higher than 15% inhibition. These results indicate that antibodies against the toxin binding site exist. In contrast, there are no antibodies against the hexamethonium or the carbamylcholine binding site because no inhibition of ^{125}I-anti-AcChR binding is observed with these two agents. It is quite possible that, in these two latter cases, the inhibition is too small and masked by high nonspecific binding.

Nonspecific binding of ^{125}I-anti-AcChR antibodies to membrane fragments can be explained by at least three different mechanisms which could be simultaneously involved:

1) the antibodies have been damaged by the iodination procedure.

2) Iodinated IgG bind to the membrane fragments through hydrophobic forces.

3) Acidic mucopolysaccharides still bound to the fragments bind IgG through electrostatic forces.

The relative importance of these different mechanisms were further studied:

Experiment A (see results and Fig. 5) indicates that the anti-AcChR antibodies have been damaged by the iodination procedure because:

1) unlabeled anti-AcChR antibodies inhibit only 70% of the binding of the labeled antibodies
2) a more than 10 fold excess of unlabeled antibodies is necessary to decrease the binding of the labeled antibodies by 50%.

Experiment B (see results) indicates a nonspecific binding of antibodies to membrane fragments:

3-12% of labeled control sheep-anti-rabbit IgG antibodies bind to the membrane fragments.

This can be due to the iodination procedure.This experiment also shows that α-Bgt, while inhibiting ^{125}I-anti-AcChR binding by 10-15%, does not inhibit ^{125}I-SARIG binding to the membranes. The inhibition of ^{125}I-anti-AcChR binding observed with α-Bgt can therefore be considered as specific.

Thus, a small subpopulation of antibodies directed against the toxin binding site is present in the sera tested. We attempted to purify antibodies directed exclusively against the toxin binding site as these antibodies and those directed against other sites of the AcChR could be used separately to try to transfer EAMG. In a first step, we used ^{125}I-anti-AcChR antibodies, as we did not know whether or not we could obtain a sufficient quantity of purified antibodies to characterize them (they should be checked for their ability to inhibit ^{125}I-α-T binding to AcChR-rich membrane fragments). We adsorbed these labeled antibodies on membrane fragments and measured the radioactivity released in presence of different agents. Fig. 6 shows that 2M NaCl releases 50% of the ^{125}I labeled antibodies from the membranes. Thus, 50% of the labeled antibodies were nonspecifically adsorbed. 3 to 7% of the ^{125}I-anti-AcChR antibodies were displaced by α-T, α-Bgt or hexamethonium. As only 3% of the ^{125}I-IgG incubated are adsorbed on the fragments and only about 5% of these adsorbed antibodies can be desorbed with toxin, the amount of ^{125}I-anti-AcChR competing for the toxin binding site represents only 0.15% of the total amount of ^{125}I-IgG incubated. Accordingly, if we start with 100 gr of *Torpedo* electric organ (about 10 nmoles of ^{125}I-α-Bgt binding activity) and adsorb 4.5 mg anti-AcChR antibodies (see results), we will be able to desorb only about 5 to 10 µg specific antibodies with the neurotoxin. As one electric organ of *Torpedo* weighs between 50-150 gr, we would need 10 organs to purify 50-100 µgr of specific antibodies. Thus we decided not to start with such a large scale purification

and tried, instead, to separate anti-AcChR antibodies directed against parts of the AcChR accessible to antibodies when the receptor is in its membrane environment, from antibodies directed against parts of the AcChR hidden within the membrane. The antisera should contain these two types of antibodies since the rabbits are immunized with detergent-solubilized AcChR. The antibodies were adsorbed on an adequate amount of membrane fragments. The anti-AcChR antibodies remaining in the supernatant no longer inhibited ^{125}I-anti-AcChR binding to the fragments. This could have indicated that all the antibodies directed against parts of the AcChR not hidden within the membrane (hydrophilic antigenic sites) had been adsorbed. But when the antibodies from the supernatant were quantified by radioimmunoassay, it was found that there was a continual decrease in the amount of anti-AcChR present during successive adsorptions with membrane fragments. The antibody titer should have reached a plateau and not decreased linearly if only antibodies against hydrophilic parts of the AcChR had been specifically adsorbed on the membranes and if the radioimmunoassay also measures antibodies directed against antigenic sites of the AcChR usually hidden within the membrane. Thus it seems that anti-AcChR antibodies were adsorbed both specifically and nonspecifically on the membrane fragments. We did not succeed in decreasing this nonspecific adsorption by treating the membranes with 1M NaCl after adsorption or by preincubating the fragments with a large excess of unrelated immunoglobulins.

Another way of explaining the progressive disappearance of anti-AcChR antibodies from the supernatant in the course of successive adsorptions on membrane fragments is that the radioimmunoassay measures only antibodies directed against hydrophilic parts of the AcChR. The hydrophobic antigenic sites are not accessible to the antibodies since they are surrounded by detergent molecules. If this were true, there is no way of distinguishing between two populations of anti-AcChR anti-

bodies, that directed against parts of the AcChR accessible to antibodies (hydrophilic antigenic sites) and that directed against parts of the AcChR hidden within the membrane (hydrophobic antigenic sites).

We thus gave up the purification of different subpopulations of anti-AcChR antibodies. In the conclusion, we describe experiments which could be performed to check which antigenic sites of the AcChR molecule (that is, what subpopulation of anti-AcChR antibodies) are essential to induce EAMG.

CONCLUSION

The main purpose of this work was to analyse whether the anti-AcChR antibodies present in the sera of rabbits immunized with purified *Torpedo* AcChR played a role in the appearance of signs of paralysis observed in EAMG. On one hand, as shown by Lindstrom (1976a) and in paper (2), there is no correlation between the amount of anti-AcChR antibodies detected by RIA and the clinical status of the animal. On the other hand, we found a positive correlation between the inhibition of ^{125}I-α-T binding to solubilized AcChR produced by anti-AcChR antibodies and the appearance of signs of paralysis in an immunized rabbit (paper (2)). We suggested that a subpopulation of antibodies directed against the toxin binding site of the AcChR might play a role in the appearance of the neuromuscular blockade.

Several authors have analysed the effect of anti-AcChR antibodies or myasthenic sera on toxin binding to the AcChR:

a) Lindstrom (1976d) showed that the number of α-Bgt binding sites per eel electrocyte decreases by 50% after incubation of the cells with anti-eel AcChR serum.

b) Pre-incubation of solubilized rat AcChR with myasthenic serum inhibited ^{125}I-α-Bgt binding by 50% (Almon and Appel, 1975).

c) Bender et al. (1975) demonstrated on normal human muscle sections a complete inhibition of α-Bgt binding with myasthenic sera.

Theoretically the inhibition of toxin binding could be due to specific antibodies directed against the toxin binding site or/and to a steric or allosteric effect of antibodies directed against antigenic sites different from the toxin binding site. According to Lindstrom (1976d) and Almon and Appel (1975) the inhibition of toxin binding is due to an allosteric or steric effect and not to antibodies acting directly at the toxin binding

site.

The results of our experiments, however, suggest that, besides antibodies directed against other sites of the AcChR and exerting an allosteric or steric effect on toxin binding, there is a small subpopulation of anti-AcChR antibodies directed against the toxin binding site itself of the AcChR. This indicates that the toxin binding site might be essential for inducing EAMG. This would have been definitely proven by the successful transfer of EAMG with this specific subpopulation of anti-AcChR antibodies but we failed in our attempts to purify it.

Another approach to this problem could be used to analyse whether or not the toxin binding site of the AcChR is essential to induce EAMG: experiments based on chemical modifications of the AcChR with specific agents such as MBTA which covalently binds to the acetylcholine binding site.

The neurotoxins are widely used as markers of the AcChR. They completely inhibit AcCh binding to AcChR and block the electrophysiological response of muscle cells to either nerve stimulation or iontophoretically applied AcCh. But this does not mean that the molecular structures of the AcCh and the toxin binding sites are identical. AcCh is a small molecule with a molecular weight of 150 daltons whereas neurotoxin is a protein of about 8.000 daltons. It is therefore likely that the neurotoxin, besides binding to the AcCh binding site, also binds to other sites on the AcChR.* Furthermore, it is still not certain whether or not the number of AcCh and toxin binding sites per AcChR molecule is identical. There is evidence showing that there are

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* Valderrama et al. (1976) compared ^{3}H-MBTA and ^{3}H-toxin labeled AcChR as antigens in the radioimmunoassay. They showed that the titer of anti-AcChR antibodies in rabbit sera is greater against ^{3}H-MBTA than ^{3}H-toxin labeled AcChR. This is consistant with the fact that the toxin molecule masks more antigenic determinants on the AcChR than does MBTA (molecular weight = 372).

twice as many toxin binding site than AcCh binding sites (Fulpius 1976). The fact that anti-AcChR antibodies inhibit toxin binding to the AcChR does therefore not necessarily imply that these antibodies also inhibit AcCh binding. Our experiments on mouse diaphragm indicate that the anti-AcChR sera contained antibodies against the toxin binding site and not against the AcCh binding site: (i) anti-AcChR serum inhibits ^{125}I-α-T binding to mouse diaphragm, (ii) anti-AcChR antibodies do not decrease the amplitude of contraction of a rat nerve-diaphragm preparation

Other investigators, using more sensitive electrophysiological recordings, have reported an effect of anti-AcChR antibodies on the response to AcCh and carbamylcholine: Anwyl et al. (1977) and Bevan et al. (1977) showed on muscle cells in culture, that the IgG present in myasthenic serum decreases the sensitivity of AcChR to iontophoretically applied AcCh. Patrick et al. (1973b) and Sugiyama et al. (1973) demonstrated, using eel electroplax, that anti-AcChR serum decreases the response to applied carbamylcholine. Finally, Green et al. (1975) recorded the MEPP in frog muscle endplates and noticed that their amplitude decreased after incubation in presence of anti-AcChR serum.

Accordingly, it seems well established that the anti-AcChR antibodies are able to block the electrophysiological response of muscle to AcCh. However, this does not mean that they do so by preventing AcCh binding to AcChR.

We can conclude from our experiments and from published data (Table II) that the anti-AcChR antibodies present in myasthenic sera and in the sera of animal immunized with AcChR play a major role in the neuromuscular blockade observed in myasthenia. It was interesting to know by what mechanism these antibodies induce neuromuscular blockade.

Three different mechanisms can be considered:

TABLE II

Arguments favoring a role of anti-AcChR antibodies in producing a neuromuscular blockade in MG and EAMG

1) Inhibition of ^{125}I-α-Bgt binding to solubilized rat AcChR in presence of myasthenic serum (Almon and Appel, 1975)

2) Inhibition of ^{125}I-α-T binding to solubilized AcChR during immunization of a rabbit (paper(2))

3) Inhibition of ^{125}I-α-Bgt binding to human muscle sections in presence of myasthenic serum (Bender et al. 1975)

4) Inhibition of ^{125}I-α-T binding to *Torpedo* membrane fragments in presence of myasthenic immunoglobulins (Lefvert and Bergstrom, 1977)

5) Inhibition of ^{125}I-α-Bgt binding to *Electrophorus electricus* electroplax in presence of anti-eel AcChR serum (Lindstrom, 1976d)

6) Inhibition of ^{125}I-α-T binding to mouse diaphragm in presence of anti-*Torpedo* AcChR antibodies (paper (2))

7) Block of electrophysiological response to applied carbachol in presence of anti-eel AcChR serum in *Electrophorus electricus* electroplax (Patrick et al., 1973, Sugiyama et al., 1973)

8) Decrease in MEPP amplitude in frog endplate in presence of anti-*Torpedo* AcChR serum (Green et al., 1975)

9) Increased degradation rate of AcChR in muscle cells in culture in presence of myasthenic immunoglobulins and anti-eel AcChR antibodies (Appel et al., 1977, Kao and Drachman, 1977, Heinemann et al., 1977)

TABLE II (continued)

10) Decrease in sensitivity to iontophoretically applied AcCh on muscle cells in culture in presence of myasthenic serum (Anwyl et al., 1977, Bevan et al., 1977)

11) Passive transfer of myasthenic symptoms to an animal with myasthenic immunoglobulins and anti-eel AcChR antibodies (Toyka et al., 1975, Lindstrom et al., 1976c)

12) Presence of immunoglobulins and C_3 component of complement in myasthenic neuromuscular junctions (Engel et al., 1977)

1) the antibodies produce a pharmacological block of the AcChR (the anti-AcChR antibodies act as curare-like agents) by binding to the AcCh binding site of the AcChR,

2) the AcChR are degraded after internalization (modulation) of the anti-AcChR-AcChR complexes,

3) the AcChRs are destroyed after activation of the complement reaction which is induced by binding of the anti-AcChR to the AcChR. This does not need to occur at the toxin binding site.

From the data of paper (2), anti-AcChR antibodies inhibit ^{125}I-α-T binding to the neuromuscular junction region of mouse diaphragms in absence of complement and at 4°C (temperature at which modulation of membrane proteins is not possible). Thus, in this case, anti-AcChR antibodies interact with the toxin binding site of the AcChR. This favors mechanism 1).

Mechanism 2) is illustrated by experiments performed on muscle cells in culture. Kao and Drachman (1977) and Appel et al. (1977) showed that myasthenic serum increases the degradation rate of AcChRs, probably by inducing internalization (modulation) of the AcChR*. One should, however, remember that modulation in response to binding of anti-AcChR antibodies could be a mechanism restricted to cells in culture. To exclude the possibility that the anti-AcChR antibodies induce a modulation of the AcChR, one could passively transfer monovalent anti-AcChR F_{ab} fragments to an animal instead of intact divalent IgG.

Mechanism 3) is illustrated by experiments performed by Engel et al. (1977). They demonstrated the presence of IgG and the C_3 component of complement in the neuromuscular junction of myasthenic

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* In addition, Anwyl et al. (1977) and Bevan et al. (1977) postulate that the decrease in sensitivity to iontophoretically applied AcCh they observe after incubation of the muscle cells with myasthenic serum is due to a decrease in the number of AcChR on the myotubes and not to a pharmacological block of the AcChR (the decrease in AcCh sensitivity is temperature dependent).

patients by using peroxidase-labeled protein A (protein A from Staphylococcus aureus which binds specifically to the F_c portion of IgG) and rabbit anti-human C_3.

There is an apparent discrepancy between these latter results and our results on mouse diaphragms but it is possible that the anti-AcChR serum we used to inhibit ^{125}I-α-T binding to mouse diaphragm contains antibodies which *in vivo* would bind complement but that they also contain a small population of anti-AcChR antibodies which pharmacologically block the AcChR. Only these latter antibodies would have been active in our experimental conditions. As Engel et al. stated in their paper, the fact that they found IgG and C_3 in the neuromuscular junctions of myasthenic patients does not exclude a contribution of a pharmacological blockade and of IgG-induced modulation of the AcChR in the production of the disease. The only way to know whether the complement is involved in the appearance of the neuromuscular blockade would be to immunize complement-deficient rats or rabbits with AcChR or to inject them with anti-AcChR antibodies and check whether they become paralysed.

During 1977 several reports have appeared which all attribute an important role to the anti-AcChR antibodies in the production of the neuromuscular blockade observed in myasthenia. It thus seems that the original hypothesis put forward by Simpson in 1960 that myasthenia gravis is an autoimmune disease in which an antibody to endplate protein may be formed is verified. But it is still a matter of debate by what immunological mechanism the anti-AcChR antibodies exert their action. The transfer of anti-AcChR F_{ab} fragments into mice (F_{ab} fragments can neither bind complement nor induce modulation of the AcChR) and the immunization of complement-deficient animals with AcChR will be the best experiments to perform to find an answer to this last question.

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